Simultaneous Removal of Emerging Organic Contaminants, COD, and Nutrients by a Bio-Fenton SBR

バイオフェントン SBR による新興有機汚染物質、COD および栄養塩類の同時除去

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Waseda University Graduate School of Creative Science and Engineering

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Abstract

The intensive usage of antibiotics and the inadequate removal of such emerging organic contaminants (EOCs) from wastewater have caused the spread of antimicrobial resistance in bacteria, leading to a decline in the effectiveness of antibiotics used to treat diseases in humans and animals. Activated sludge-based conventional wastewater treatment plants (WWTPs) are generally designed to remove conventional pollutants and nutrients while not capable of removing these refractory contaminants to an acceptable level. Fenton reactions depending on the production of reactive oxidizing species like hydroxyl radicals (·OHs), hold great promise for decomposing recalcitrant organic pollutants. The drawbacks of classic Fenton reaction, including the generation of iron sludge, the need for acidic environment, and the expensive cost of Fenton reagents (e.g., hydrogen peroxide H_2O_2), have restricted its practical applicability. The main aim of this research is to treat antibiotics effectively and sustainably without sacrificing the ability to remove chemical oxygen demand (COD), and nutrients such as nitrogen and phosphorus. A Bio-Fenton sequencing batch reactor (SBR) was proposed, where anaerobic and aerobic activated sludge were mixed with magnetite and subjected to an alternative anaerobic/aerobic condition. Long term experiment was conducted to support the hypothesis that mixed microbial cultures in activated sludge could produce *in-situ* H_2O_2 for use in Bio-Fenton reaction. The supplemental magnetite might function as the heterogeneous Fenton catalyst, which is reduced to produce Fe (II) in an anaerobic environment, and the Fe (II) participates in the Bio-Fenton reaction and is subsequently oxidized to Fe (III) in an aerobic environment. From this point of view, the continuous redox cycle of magnetite and the ongoing generation of H_2O_2 by bacteria might maintain the continuous occurrence of Bio-Fenton reaction, and the resulting ·OHs can effectively decompose the model antibiotic sulfamethoxazole (SMX). The

reactors were operated in a neutral environment under various hydraulic retention time (HRT) and magnetite dosage settings to investigate their removal performances. The results of the experiment indicate that stable and enhanced SMX removal was obtained in the Bio-Fenton SBR with 1 g/L of magnetite under the HRT of 4, 2, and 1 day, with the maximum removal efficiency reaching 100%, as opposed to just 60% in the control SBR. The removal of conventional organic pollutants was unaffected by the inclusion of magnetite and the SBRs with and without magnetite were all able to eliminate more than 80% of COD. H_2O_2 was produced by the microorganisms and was found both in the sludge flocs and in the bulk phase. Approximately 50 μM of H_2O_2 was detected in the bulk phase of the Bio-Fenton SBRs under an HRT of 4 days, which was about 30 μM greater than that in the SBR without magnetite. The amount of H_2O_2 presented in the liquid phase became smaller when the HRT was shortened. Alternating anaerobic/aerobic conditions resulted in a continuous redox cycle of magnetite, while the quantity of the reduced and oxidized magnetite decreased along with the lowered HRT, demonstrating a pattern that was comparable to that of H_2O_2. In order to explore the primal mechanisms of both COD and SMX removals, COD balances and the formation of ·OHs, respectively, were analyzed and detected. During anaerobic periods in the Bio-Fenton SBR supplemented with 1 g/L of magnetite, denitrification, phosphate release, and microbial Fe (III) reduction were major processes. The production of ·OHs was identified by employing a novel fluorescence reagent known as APF, and a confocal laser scanning microscopy was utilized for observing the fluorescence. Strong fluorescent emissions were observed in the sludge flocs withdrawn from the Bio-Fenton SBRs under the HRT of 4 and 2 days, during which the microbial H_2O_2 production and redox of magnetite were also greater, implying the creation of ·OHs. However, under fluorescent irradiation, extremely weak or no fluorescence was observed in the sample from SBR which did not contain additional magnetite. The stable and enhanced SMX removal in the Bio-

Fenton SBRs during certain phases was revealed to be due to the formation of ·OHs via biological Fenton reaction, which took place in the matrix of magnetite-sludge aggregates. Our work involving studies of microbial production of H_2O_2 in activated sludge as well as the occurrence of Bio-Fenton reaction prove to be encouraging for the further effective removal of contaminants of emerging concern.

List of abbreviations

Advanced oxidation processes (AOPs)

Antimicrobial resistance (AR)

Chemical oxygen demand (COD)

Dichloro-diphenyl-trichloroethane (DDT)

Emerging contaminants (ECs)

Emerging organic contaminants (EOCs)

Endocrine-disrupting compounds (EDCs)

Flavin adenine dinucleotide (FAD)

Glucose oxidase (GOD)

Hydraulic retention time (HRT)

Iron-reducing bacteria (IRB)

Lactic acid bacteria (LAB)

lipoamide dehydrogenase (Lpd)

Mixed liquor suspended solids (MLSS)

Mixed liquor volatile suspended solids (MLVSS)

Pentachlorophenol (PCP)

Phosphorus accumulation organisms (PAOs)

Highly reactive oxidative species (hROS)

Sequencing batch reactor (SBR)

Sludge volume index (SVI)

Sulfamethoxazole (SMX)

Superoxide dismutase (SOD)

Wastewater treatment plants (WWTPs)

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Chapter 1: Research background

1.1 Occurrences of emerging organic contaminants

The rise in global population over the past few decades, along with the concomitant expansion of urban, industrial, and agricultural sectors, has led to an increase in the negative impact that human activities have on water resources. This trend has been observed in both developed and developing nations (Iwu et al., 2020). Water pollution from various harmful and non-biodegradable organic pollutants has grown into a severe issue that has to be addressed for the sake of the environment and the welfare of people. Even in sparsely populated areas, the overuse of water resources and pollution of water bodies are serious problems that have an adverse influence on both the ecology and public health. There are a variety of geological processes and anthropogenic factors that can contaminate the water. One of the primary sources of water contamination corresponds to direct release of both treated and non-treated wastewater from urban, industrial as well as agricultural sectors (Burri et al., 2019; Singh et al., 2019). Recently, the usage of new compounds and additions in a variety of producing sectors has caused the presence of components and molecules in sewage effluents. As a result, emerging contaminants (ECs), which consist of a variety of organic and/or inorganic substances, have negatively impacted aquatic and terrestrial organisms, as well as human health. (Lozano et al., 2022).

Emerging organic contaminants (EOCs), the largest group of ECs, are found in a broad range of products that are used on a daily basis, for example, pharmaceuticals, personal care products, and other items. They are discharged into sewage systems via human excrement, laundering, bathing, and flushing expired or unused drugs (Tran et al., 2018). Pharmaceuticals and personal care

products (s) are the most prevalent categories of ECs. Pharmaceuticals include antibiotics, antimicrobial, hormones, and other illegal drugs. While personal care products include food additives, cosmetics, biocides and some other personal sanitary items (Pai & Wang, 2022). Besides these molecules, intermediates and byproducts of PPCPs are also regarded to be EOCs (Fu et al., 2019). These natural or synthetic compounds (e.g., EOCs) are attributed to a potentially hazardous effect on both human health and the environment. Form a source-to-receptor point of view, Figure 1 shows how these emerging pollutants move through their life cycles.

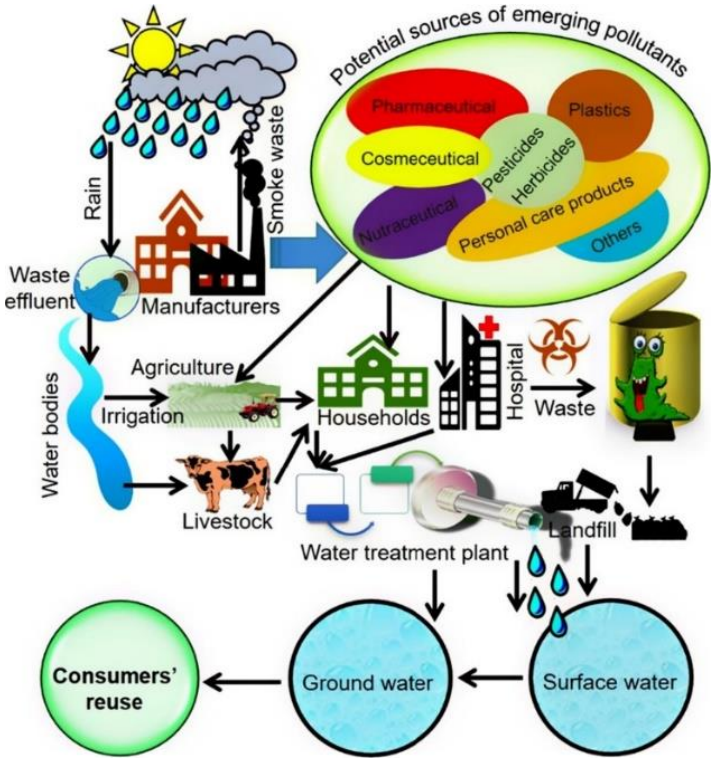

Figure 1.Source-to-receptor life-cycle dispersion of ECs (Rasheed et al., 2019). Adapted from *Environment international*.

1.2 Antibiotics and antimicrobial resistance

Antibiotics as one of the most popular groups of EOCs, are chemicals that prevent or suppress the growth of microbial with little or no harm to the host. In 1928, Alexander Fleming unintentionally discovered penicillin after seeing that the growth of Staphylococcus was inhibited by a substance released by Penicillium notatum. It was further refined for application by Florey and Chain, and this proceeded the invention of several types of penicillin (Penesyan et al., 2015). Antibiotics were then classified as “natural compounds of microbial origin” by Selman Waksman after he found that the metabolites yielded by bacteria and fungus that were isolated from this environment could effectively cure human infections with few side effects. During the “golden period of antimicrobial discovery” which lasted from the 1950s through 1970s, newer classes of non-synthetic antibiotics were created. But by the middle of 1960s, it was tougher to use this paradigm to produce newer and much more potent antimicrobial. This was principally due to the fact that particular environments were required for the growth of microbial that produced antibiotics. As a consequence of the pharmacological and toxicological issues that came with these antibiotics, the resistance to the early antimicrobial emerged. This led to the start of the era of synthetic variants of antibiotics, which were effective in preventing the antimicrobial resistance (AR). However, after some period of time, these synthetic antibiotics began to show downward pleiotropic and complicated impacts on microorganisms, compelling bacteria to develop adapted survival methods (Brown & Wright, 2016). Antibiotics are now regarded as endangered species since they are being phased out of existence as a result of the widespread prevalence of AR (Founou et al., 2016).

In most cases, when antibiotics are taken over abundance, the drug-vulnerable bacteria are removed, leaving the drug-resistant bacteria to thrive by natural selection (Read & Woods, 2014). However, in spite of the warnings

about their abuse, antibiotics are overused globally, resulting in the dramatic decrease in the discovery of novel antibiotic that combat development of AR (Wendlandt et al., 2015). Universally, the annual consumption rate of antibiotics is estimated to vary between 0.1 and 0.2 million tons. While in 2010, only livestock consumed about 63,151 tons of antibiotics worldwide, and if nothing is done with this issue, this consumption is projected to increase by 67% by the year 2030 (Van Boeckel et al., 2015). After antibiotics enter the human or animal body, 30% ~ 90% of them are excreted via urine and feces in the form of maternal structure or metabolites, hence, sewage is considered as a main route for antibiotics to reach the environment (Lin et al., 2009). Unfortunately, wastewater treatment plants (WWTPs) that employ physical, chemical, biological, and disinfection processes are adopted to reduce conventional organic pollutants and pathogens prior to discharge. Sufficient treatment of ECs is not conducted in most WWTPs. Most ECs can pass through WWTPs because of their refractory characteristics and continuous introduction (Bolong et al., 2009). According to the predicted statistical models reported by Resistance, 2014 and Antimicrobial Resistance, 2022, there were an anticipated 4.95 million fatalities attributed to AR in 2019, and it indicates a continuous growth in AR would lead to 10 million deaths annually by 2050.

1.3 Treatment of emerging organic contaminants

1.3.1 Biological process in conventional WWTPs

As reflected in the Sustainable Development Goal (SDG) goal 6, improving water quality and sanitation are the most pressing issues facing in humanity in the 21st century. Despite the fact that WWTPs' overarching objective is to minimize the load of contaminants reaching waterbodies, they often put emphasis on the removal of conventional organic pollutants and nutrients rather

than refractory pollutants of emerging concern. And thus, one of the concerns that is becoming increasingly critical when it comes to wastewater treatment is the enhanced removal of particular contaminants, such as the EOCs discussed above (Habitat & Organization, 2018). Biological process such as activated sludge process plays a significant role in reducing harmful organic pollutants in wastewater and it is the most frequently used in WWTPs. Due to the fact that some EOCs like antibiotics in wastewater are present in trace amounts and have antibacterial properties, activated sludge process exhibits poor removal performance. To improve the efficiency of removing these trace toxic organic contaminants, numerous efforts have been made to modernize the biological treatment techniques, for instance, biological acclimation, which enhances the removal efficiency by enriching the predominant microorganism species (Jianlong et al., 2002). In biological treatment system, the metabolic processes carried out by microbes have an influence on removing organic pollutants. It may be challenging to establish favorable environments for the promotion of microbial activity when poisonous by-products are present. Additionally, the capability of microorganisms to oxidize organic pollutants to enhance the biodegradability of wastewater is further hindered by the insufficient biodegradation of organics (Bahri et al., 2018; Nidheesh et al., 2021).

Significant quantities of antibiotic residues have been detected in WWTPs and in effluent-receiving waterbodies as a consequence of the widespread usage of antibiotics. Despite the seriousness of the problem, traditional WWTPs are not presently built to remove these EOCs probably because of the cost the limitation of technology development. According to research, WWTPs are only capable to successfully removing 20% to 90% of antibiotic residues passively via the process of sludge adsorption and the natural decompose of several antibiotics, like penicillin. Unfortunately, plenty other antibiotics including fluoroquinolones, tetracyclines, and sulfamethoxazole (SMX), are far more refractory to the natural degradation and thus continue to be present in the

effluent form WWTPs (Becker et al., 2016; Watkinson et al., 2007). Although biological process has advantages from both an operational and financial standpoint, and majority of WWTPs use it, it aids in the spread of AR because activated sludge has a diverse microbial community in which the enormous biomass and large variety of bacteria can create suitable habitat for enriching AR (Niestępski et al., 2020).

1.3.2 Advanced oxidation processes (AOPs)

As the environmental discharge criteria are getting increasingly rigorous, the traditional continuous flow-based biological wastewater treatment technologies confront substantial challenges. Activated sludge technology has been undergoing several minor and major modifications over the past few years, at the same time, more effective alternatives have also been introduced to treat the continuously growing number of new pollutants in wastewater. The utilization of advanced oxidation processes (AOPs) which are regarded as very promising alternative treatment strategies for dealing with environmental challenges related to pollutants of emerging concern, has attracted considerable interest in recent years. Oxidizing agents produced in AOPs, such as hydroxyl radicals (·OHs), are able to react non-selectively with a broad variety of organic compounds, therefore degrading and mineralizing their structures into nonhazardous molecules like carbon dioxide, water, and inorganic ions. Different AOPs, including Fenton-based reactions, ozonation, photocatalysis, etc. are commonly researched because they can converse the pollutants into much easier biodegradable intermediates or completely mineralize the contaminants (Hayati et al., 2020; Wang & Xu, 2012).

Fenton-based reactions are seen as potentially efficient and cost-effective methods referring to a group of reactions of peroxides with iron ions to produce reactive oxidizing species for decomposing recalcitrant organic pollutants

(Pignatello et al., 2006). The widely recognized mechanism of classic Fenton reaction (homogeneous Fenton process) is a series of chemical reactions that use Fe^{2+} or Fe^{3+} as catalyst to breakdown hydrogen peroxide (H_2O_2), thus triggering the formation of hydroxyl radicals in an acidic media [reaction (1) and (2)] (Barb et al., 1949). When Fe^{3+} is utilized as the catalyst to decompose H_2O_2, this reaction is known as Fenton-like reaction (Barb et al., 1949; Siedlecka et al., 2008). No matter which ion is used to trigger the reaction, it can be evident from the preceding reactions that both Fe^{2+} and Fe^{3+} are available concurrently. Therefore, the reactions lunched by ferrous and ferric ions were referred as homogeneous Fenton reaction.

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH \quad k_1 = 40\sim80\ L\cdot mol^{-1}\cdot s^{-1} \qquad (1)$$

$$H_2O_2 + Fe^{3+} \rightarrow Fe^{2+} + OH^- + HO_2^{\cdot} \quad k_1 = 9.1\times10^{-7}\ L\cdot mol^{-1}\cdot s^{-1} \qquad (2)$$

Where, k_1 and k_2 indicate their constant rate, respectively.

There are a few drawbacks associated with homogeneous Fenton processes, such as the need for additional iron sludge dispose, the cost and dangers of storing and transporting H_2O_2, and the requirement for acidic (pH 2-4) conditions (Brillas et al., 2009). To address these shortcomings of homogeneous Fenton reaction, solid iron-based catalysts including iron oxides like Fe_2O_3 and Fe^0/Fe_3O_4, zero-valent iron, and iron minerals, have been introduced to heterogeneous Fenton reactions, which have garnered a lot of interest as of late because they can catalyze the Fenton reaction in a neutral pH environment (Deng et al., 2008; Lee et al., 2008; T. Li et al., 2021; Liu et al., 2021; Zhao et al., 2021). Generally, the oxidation of organic contaminants takes place either by the iron ions released to the bulk phase or by the interactions between dissolved substances and surface-bound compounds (Brillas et al., 2009).

1.3.3 Combined biological treatment and AOPs

There is currently no single unit technique for the effective as well as sustainable treatment of these EOCs since conventional WWTPs cannot degrade these pollutants to a sufficient level. Therefore, there is an increasing interest in researching how to combine several treatment methods for the greatest operational flexibility. To this date, different of modified AOPs or a combination of biological process and AOPs have been developed and used for the treatment of target compounds in a variety of matrices with the specific intention of improving the degradation performance. The potential benefits of integrating biological processes with AOPs seem to be an appealing option for removing persistent organic pollutants on the premise that microorganisms are readily use a number of oxidation products from these pollutants (Nidheesh et al., 2021; Ouarda et al., 2018).

Several studies have assessed the performance of combining AOPs and biological treatments in sequence, for example AOPs either as a pretreatment or a posttreatment, for the removal of certain antibiotics from aqueous system (Belgiorno, 2013; Khan et al., 2019; Moussavi et al., 2019). According to the findings of some research, the biodegradability of the effluent from advanced oxidated treatment had been significantly improved. The treatment of resistant wastewater, such as semiconductor wastewater (Lin & Jiang, 2003), azo dyes wastewater (Tantak & Chaudhari, 2006), and pharmaceutical wastewater (Elmolla & Chaudhuri, 2012) was found to be successful using a Fenton process followed by a sequencing batch reactor (SBR) process. In the combined Fenton-SBR system, the by-products from degradation caused by the Fenton pretreatment are more easily biodegradable, therefore improve the effectiveness of the SBR process. In addition, there are some studies in terms of the treatment of antibiotics by developing a modified Fenton-based process. Gonzalez et al., 2007 employed the photo-Fenton process to treat a 200 mg/L

SMX solution before looking into the treated effluent's biodegradability. They were able to completely eliminate SMX at a certain H_2O_2 concentration, and the treated solution had better biodegradability. By using the solar photo electro-Fenton approach, Murillo-Sierra et al., 2018 effectively mineralized the SMX solution, and they observed that the main by-products of the biodegradation of SMX were maleic, oxalic, and fumaric acids, which are easily to be degraded in the subsequent biological treatment process. Although these combined technologies improved the removal rate and even completely removed some trace toxic organic contaminants, it is unsustainable because the ongoing need for Fenton reagents (e.g., H_2O_2 and Fe^{2+}) is still necessary.

To address these issues mentioned above, the concept of biologically producing H_2O_2 in-situ emerged and has been investigated by some researchers. For example, one method involves isolating bacteria which are capable of producing H_2O_2 via respiration. This microbially produced H_2O_2 may has the potential to be used for a Fenton process to decompose some persistent organic pollutants. Such type of procedure is referred to as the Bio-Fenton process, and this will be covered in Chapter 2.

1.4 Research objectives

There are three main objectives in this study as follows:

(i) To understand the process of microbial H_2O_2 production and current situation on the application of Bio-Fenton process to the removal of organic pollutants.

To achieve this objective, literature review was conducted to obtain a comprehensive understanding on the microbial production of H_2O_2. Moreover, the recent studies in terms of Bio-Fenton process which investigated using the H_2O_2 generated in-situ for a Fenton-based reaction were referred to by doing literature research.

(ii) To investigate the simultaneous removal of a model antibiotic, SMX and COD via the proposed Bio-Fenton SBR.

To achieve this objective, an SBR supplemented with magnetite was designed and was operated in a manner that alternated between anaerobic and aerobic conditions over an extended period of time. This allowed the reactor to reach a steady state. Under the steady-state condition, besides the removal of COD and SMX, the microbial production of H_2O_2 and the potential utilization of the solid catalyst, magnetite, were also investigated.

(iii) To identify the primal processes and removal mechanisms in the proposed Bio-Fenton SBR.

To achieve this objective, the time course variations of conventional nutrients including nitrogen and phosphorus, and dissimilatory ferric reduction that involved in the processes which contributed to COD removal were monitored. Additionally, the formation of hydroxyl radicals was detected to prove the occurrence of Bio-Fenton reaction in our proposed Bio-Fenton SBR.

Considering that antibiotics are difficult to be biodegraded, they are easily enriched in organisms and cannot be removed, which requires an effective way to oxidize them for further complete removal. Therefore, research of an effective and sustainable antimicrobial wastewater treatment process is important. At present, advanced oxidation process is a topic that is much more discussed, among which Fenton reaction is especially widely studied. However, some drawbacks of Fenton reaction, such as the acidic environment requirement, the continual demand for Fenton reagents (e.g., H_2O_2), have yet to be addressed. Bio-Fenton process offers solutions to these limitations, since it is capable of producing H_2O_2 in-situ and can occur in a neutral environment. The in-situ generation of H_2O_2 is a crucial determinant in the occurrence of Bio-Fenton reaction. It is essential to comprehend the potential pathways of H_2O_2 production. On the other hand, the removal efficiencies achieved by the Bio-Fenton reaction are highly dependent on the particular target pollutants that are

being employed. The current scenario of applying Bio-Fenton process in a variety of situations needs to be reviewed, which comes to the first objective of this research.

In WWTPs that incorporate activated sludge process, the removal of conventional pollutants (including biodegradable organic matters, total suspended solids, fecal coliform, and oil and grease) and conventional nutrients (including nitrogen and phosphorous) (Viessman & Hammer, 1993) is frequently prioritized above the removal of refractory contaminants such as antibiotics. Therefore, the performance of activated sludge process needs to be enhanced. The integration of activated sludge process and Fenton reaction in a sequencing batch reactor was carried out to improve the removal of antibiotics. Magnetite was used as the heterogeneous Fenton catalyst. The potential performance of Bio-Fenton reaction activated by magnetite and the H_2O_2 produced by mixed microbial culture in activated sludge has not been investigated, thus the second objective was placed on this issue.

In addition, in conjunction with the observed performance of the proposed Bio-Fenton SBR, it is important to look into the primary reactions that have contributed to the removals under both anaerobic and aerobic circumstances. The third objective concentrated on the COD balance analysis and the demonstration of ·OHs formation to provide an explanation of the mechanisms behind the removal of COD and the enhanced removal of SMX. The fundamental concept of the designed Bio-Fenton SBR is illustrated in Figure 2.

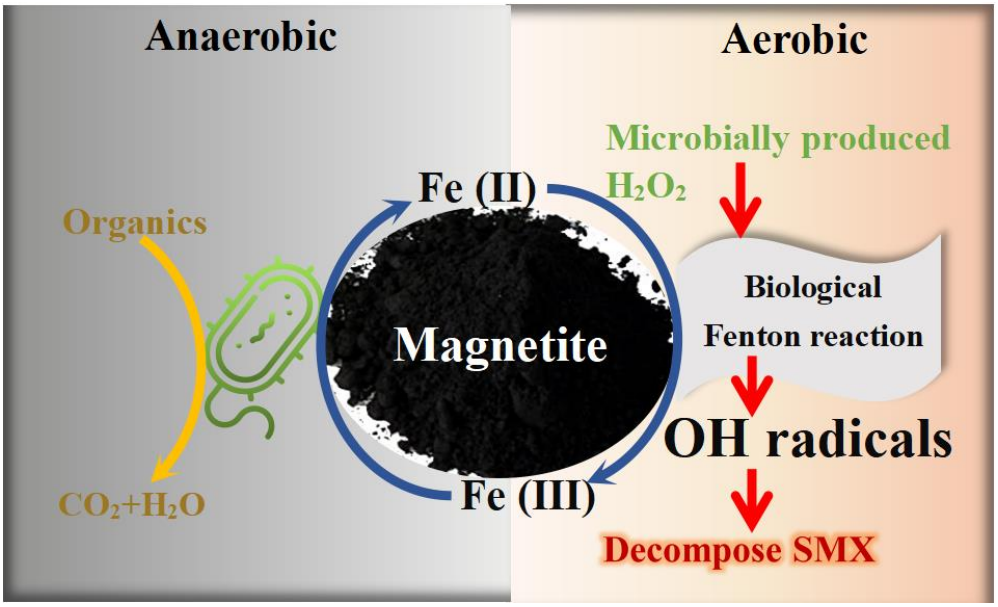

Figure 2. Illustration of the conceptual processes in the Bio-Fenton SBR.

Chapter 2: Bio-Fenton process

2.1 Introduction

To develop a Fenton process where H_2O_2 is generated in-situ, aiming to reduce the risk associated with transportation and storage, Bio-Fenton process is studied recently. Bio-Fenton is a promising approach with several additional benefits, including less energy consumption and the capability to generate H_2O_2 on-site, hence lowering overall costs. This process is a relatively recent innovation applied to wastewater treatment, and it has been studied mostly to treat effluents contain dye such as Acid blue (Eskandarian et al., 2014). Bio-Fenton process has been investigated recently in terms of employing an enzyme to catalyze a reaction that create H_2O_2 as the primary or by-product in order to provide it as Fenton's reagent. *Streptococcus oralis*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Lactobacillus johnsonii*, all of which are members of Lactobacillales order, are well-known examples of bacteria that are capable of forming H_2O_2 (Hertzberger et al., 2014). Some iron reducing bacteria, such as *Shewanella oneidensis* and *Shewanella putrefaciens* grow under both anaerobic and aerobic conditions and are capable of reducing O_2 to H_2O_2 in aerobic environment (Mckinzi and DiChristina, 1999). Several distinct enzymes, including NADH oxidase, pyruvate oxidase, α-glycerophosphate oxidase, glucose oxidase, and oxalate oxidase, are responsible for the production of hydrogen peroxide in biological systems (Condon, 1987). There are also some investigations concentrated on the microbial production of H_2O_2 produced by facultative anaerobic bacteria isolated from specific environments. These studies have given rise to the concept of introducing microbial in-situ H_2O_2 production to the activated sludge

process to initiate Bio-Fenton reaction, which would result in an enhanced removal of EOCs.

2.2 The synthesis of H_2O_2 by bacteria

2.2.1 Pathways of biological production of H_2O_2

It is well knowledge that several different species of bacteria are capable of creating H_2O_2. Although some of these bacteria are pathobionts or opportunistic pathogens such as S*treptococcus pyogenes* (Seki et al. 2004), *Streptococcus mutans* (Thomas & Pera, 1983), and *Streptococcus pneumoniae* (Pericone et al., 2003), it has been proposed that certain H_2O_2-producing strains may have beneficial effects on human health such as *Bifidobacterium bifidum* (Kawasaki et al., 2009) and *Lactobacillus johnsonii* (Pridmore et al., 2008). In detail, H_2O_2 is mainly produced in the core carbon and energy metabolism of the cells by oxidases, including NADH oxidases [reaction (3)], pyruvate oxidase [reaction (4)], α-glycerophosphate oxidase [reaction (5)], and glucose oxidase [reaction (6)] (Condon, 1987, Bankar et al., 2009). Additionally, H_2O_2 is a by-product of some flavoprotein autoxidation, and it is possible for this byproduct to build up in aerobic cultures of many different strains (Condon, 1983). The autoxidation of respiratory dehydrogenases mainly is responsible for the formation of O_2^-, and it is believed that the dismutation of O_2^- is a physiologically major source of H_2O_2 [reaction (6)] (Condon, 1987).

The production of H_2O_2 by NADH oxidase (NOx):

H_2O_2 oxidase was first described by Dolin in *S. faecalis*. This enzyme belongs to the family of NADH oxidases (Poole et al., 2000). It is found in the cytoplasm, has not been purified, and exhibited some degree of the instability. From oxygen dioxide, this enzyme catalyzes the synthesis of superoxide anion (O_2^-) and H_2O_2. It is assumed that O_2^- is a precursor in the creation of H_2O_2.

The production of H_2O_2 by pyruvate oxidase (POx):

Pyruvate oxidase is able to catalyze the reaction between pyruvate and molecular O_2, and it was initially shown in *L. delbrueckii* (Hager et al., 1954), after which, it has also been proven in lactobacilli (Thomas & Pera, 1983), in *S. mutans* (Carlsson & Kujala, 1984), and in *P. halophilus* (Kanbe & Uchida, 1985). Hager et al., 1954 purified this cytoplasmic enzyme and demonstrated that it contained flavin adenine dinucleotide (FAD). However, pyruvate oxidase did not produce O_2^- as a precursor in cell-free extracts of *L. plantarum*.

The production of H_2O_2 by α-glycerophosphate oxidase:

Lactic acid bacteria (LAB) including enterococci (Koditschek & Umbreit, 1969), lactobacilli (Thomas & Pera, 1983), and *P. pentosaceus* (Dobrogosz & Stone, 1962), use glycerol as an oxygen-dependent growing medium, are responsible for the process that is catalyzed by a-glycerophosphate oxidase. This enzyme has undergone certain purification phases and it is cytoplasmic and also contains FAD (Koditschek & Umbreit, 1969).

The production of H_2O_2 by glucose oxidase (GOx):

GOx as one of the most often utilized enzymes, is known to catalyze the oxidation of D-glucose to produce D-glucono-1,5-lactone. During this reaction, H_2O_2 is generated as a byproduct and released in the reaction medium. In the presence of ferrous ion, the oxidation of in-situ produced H_2O_2 was activated, producing hydroxyl radicals (Kahoush et al., 2018).

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2 \quad (3)$$

$$Pyruvate + Pi\ (phosphate) + O_2 \rightarrow acetyl - phosphate + CO_2 + H_2O_2 \quad (4)$$

$$\alpha - glycerophosphate + O_2 \rightarrow dihydroxyacetone\ phosphate + H_2O_2 \quad (5)$$

$$Glucose + H_2O + O_2 \rightarrow D - Gluconic\ acid + H_2O_2 \quad (6)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (7)$$

2.2.2 Response of microbes to H_2O_2 oxidation stress

It is possible that cell damage can be caused by H_2O_2 at certain concentrations. In the field of H_2O_2 toxicology, the basic aims are to recognize the cell damages that are brought on by H_2O_2 and to determine the level of H_2O_2 that is required to cause these injuries. Since H_2O_2 in micromolar will be degraded speedily by microbes, researchers commonly added exogenous H_2O_2 in millimolar in order to induce a duration of prolonged oxidative stress. Regretfully, this large amount may cause sorts of injury that are quite uncommon at physiologically relevant dosages. This technological challenge can be sidestepped by employing mutants that unable to scavenge H_2O_2. Unexpectedly, even a concentration as low as 1 μM of H_2O_2 was sufficient to cause damages to E. coli strain (Seaver & Imlay, 2001).

According to the findings of some studies that explored the sensitivity of organisms to H_2O_2, it was discovered that microorganisms responded to sublethal doses of H_2O_2 by triggering a protective system that allow them to survive in levels of H_2O_2 that are typically lethal to them. For instance, the protective system of enteric bacteria is comprised of a group of stress proteins, which is some way shield the cell from the deteriorating effects of H_2O_2 exposure. The cells that have been induced by H_2O_2 are significantly more tolerable to the lethal H_2O_2 concentration. Contrarily, in several of the examined cultures, facultative anaerobic bacteria did not accumulate H_2O_2 when they

were exposed to an aerobic environment. This is because the protective system of bacteria that were growing under anaerobic conditions was also activated when oxygen was introduced. As a consequence, it is unlikely that the protection of anaerobic microbes from oxygen was caused by the previously formed H_2O_2. The induction of enzymes such as NADH oxidase and NADH peroxidase during oxygen activation provides evidence that these enzymes may play a function in the response to a stressful scenario (Condon, 1987). In an early study of microorganisms, McCord et al., 1971 identified a relationship between the oxygen tolerance and the level of catalase and SOD in obligate anaerobic microbes. Their results occasionally have been broadly regarded as a proof that strict anaerobic bacteria are oxygen sensitive due to the absence of these enzymes. Also presumably, H_2O_2 and O_2^- mediate the toxic effects when they are exposed to oxygen. Yet, further research proved that this correlation was not as precise as previously thought since very a few obligate anaerobic organisms have catalase and/or SOD (Griffiths & Cooney, 2002). Moreover, it has lately been apparent that many other microorganisms use peroxidases instead of catalases to scavenge H_2O_2, and superoxide reductase instead of SOD to scavenge H_2O_2 (Massey et al., 1969). The most important point is that obviously O_2^- or H_2O_2 is not the component that stops these microbes from surviving in the air or oxygen.

2.3 Recent studies on Bio-Fenton process

Hung et al., 2019 employed Bio-Fenton to degrade octylphenol polyethoxylated ($OPEO_n$); the purified lipoamide dehydrogenase (Lpd) produced from *Pseudomonas nitroreducens* TX1 oxidized NAD(P)H and consumed O_2, which resulted in the generation of H_2O_2. The additional metals interacted with H_2O_2 to generate hydroxyl radicals, which then attacked $OPEO_n$. This study also explored the relationship between the presence of NADH and

Lpd and the production of H_2O_2. The results revealed a direct proportionality between H_2O_2 production and NADH concentration, as well as a linear relationship between H_2O_2 production and Lpd concentration. After 4 ours, over 90% of $OPEO_{15}$ was reduced by the purified Lpd enzymatic Bio-Fenton process. Yang et al., 2022 looked into the degradation of chloroacetanilide herbicides via a Bio-Fenton process assisted by glucose oxidase. At a pH of 5.5, the addition of 0.5 mM Fe (III)-citrate, 32 mM glucose, and 10 U glucose oxidase (GOD) resulted in the production of 8 mM of H_2O_2 and the degradation of more than 70% of the target pollutants (including acetochlor, alachlor, and metolachlor). Ravi et al., 2020 examined how efficiently glucose might be converted into hydrogen peroxide using GOD. When the starting concentrations of glucose and GDD were 60 mM and 1 mg/mL, respectively, there was a transformation of 71% of the glucose that resulted in the production of H_2O_2, which was acceptable for the Bio-Fenton process. Even though the Bio-Fenton process was only able to eliminate 48.4% of the model chlorinated contaminant trichloroethylene, its applicability as an in-situ remediation strategy in environments with neutral pH substantially exceeds that of the traditional Fenton method. Vaithyanathan et al., 2020 recovered GOD from sterilized biosolids slurries, and 0.55 M glucose was oxidized using 1000 U/L of the BS-based GOD to yield 216 ppm of H_2O_2. In a pH - neutral environment, the biologically generated H_2O_2 participated in the Bio-Fenton process to eliminate certain of 15 pharmaceuticals.

In addition to these enzymic-based Bio-Fenton processes, the endogenous H_2O_2 generated by microorganisms and aquatic plants involved in biological Fenton reaction was also recently studied. For the first time, Marco-Urrea et al., 2010 applied biological advance oxidation process to degrade pharmaceuticals. The white-rot fungus *Trametes versicolor* was responsible for the biological production of extracellular oxidizing species via the quinone redox cycling in the presence of Fe^{3+}-oxalate. Fenton's reagent (H_2O_2) was

consequently formed via the dismutation of the oxidizing species. Hydroxyl radicals were generated via a biological Fenton-like reaction. After 6 hours of incubation, over then 80% of the initial pharmaceutical (10 mg/L) had been degraded. Similarly, Reis & Sakakibara, 2012 found that certain aquatic plants could generate stably endogenous H_2O_2 in plant tissues even when exposed to phenolic endocrine-disrupting compounds (EDCs) over a long period of time. Based on these findings, Reis et al., 2013 supplemented fresh duckweeds biomass with iron sulfate, a source of Fe^{2+}, to remove pentachlorophenol (PCP) using the biological Fenton process, which was proceeded by endogenous H_2O_2 and adscititious ferrous ions. They found that PCP was fully degraded with the addition of 2.8 mM of Fe^{2+} whereas endogenous H_2O_2 was consumed. They also noted that greater endogenous H_2O_2 consumption associated with higher Fe^{2+} concentrations. This study showed how a biological Fenton process, which was initiated by the H_2O_2 present in aquatic plants, may be used to eliminate toxic organic contaminants that are resistant to other treatments. However, the formation of •OHs was not identified. In this regard, Inagaki et al., 2016 detected the existence of •OHs in aquatic plants in the presence of solid iron compounds (such as colloidal ferrihydrite, magnetite, and ferric-ion-exchanged zeolite). They found clear fluorescence emitted from the roots of plants, indicating the production of •OHs via Bio-Fenton occurred inside the plants. They also assessed the suitability of this Bio-Fenton reaction to the treatment of pollutant 17α-ethinylestradiol (EE2). Dang et al., 2022 employed a phyto-Fenton process to a field trial in Vietnam where magnetite and soil were mixed, then vetivers were planted. The trial site was heavily polluted with Dichloro-diphenyl-trichloroethane (DDT), and the findings indicated an improved removal rate of DDTs in the presence of vetivers and nano-magnetite. It was concluded that the endogenous production of H_2O_2 and •OHs contributed to the effectiveness of the phyto-Fenton process.

2.4 Conclusions and future perspectives

This chapter gives a understand on what Bio-Fenton process is and how H_2O_2 may be created in-situ by either bacterial process or an enzymic method. The application of this process to for example treat EOCs containing wastewater will consume less energy and lower overall expenses. H_2O_2 is mainly produced in the core carbon and energy metabolism of the cells by several types of oxidases. Although it is a fact that organisms are vulnerable to a specific level of H_2O_2, their protective system will, to some extent, prevent the cells from the injury that caused by H_2O_2. The possible pathways of microbial H_2O_2 synthesis by facultative anaerobic microbes would assist in the construction of the principal concept that underpin this research. Furthermore, some recent studies on the application of Bio-Fenton process are evaluated; while previous research mainly concentrated on the enzymatic-based Bio-Fenton reaction, and the microbial production of H_2O_2 was mainly investigated in pure bacterial culture. If we would practically incorporate the Bio-Fenton process to WWTPs, for example by integrating Bio-Fenton process with activated sludge process, it is critical to assess the H_2O_2 produced in-situ in the mixed sludge culture. Information regarding the Bio-Fenton process where reusable solid iron catalyst is used is limited, therefore, future studies are needed to identify microbial H_2O_2 production by mixed microbial culture in the presence of various heterogeneous iron catalysts, especially in an aerobic environment. Further, the long-term research on such biological Fenton process needs to be conducted in order to realize its practical application in WWTPs.

Chapter 3: Long-term Bio-Fenton SBR experiment

3.1 Introduction

Antibiotics, as one group of the EOCs have been detected in wastewater over the world and their impacts on the ecosystem have been of great concern. Furthermore, the loss of efficiency of antibiotics used for treatment of humans and animals is a direct result of the spread of antimicrobial resistance in bacteria (Laxminarayan et al., 2013). Antibiotics originate from the excreta of humans and flow into the municipal wastewater. Discharge from hospitals and runoffs from animal and aquaculture industries also serve as antibiotic sources. Originally, WWTPs are quite efficient at removing nutrients from wastewater, conventional treatment processes generally fail to eliminate such complex chemical compounds like pharmaceuticals, personal care products, surfactants or other pollutants of emerging concern. Therefore, the development of feasible, efficient, and sustainable solutions for treating wastewater containing EOCs is needed. In conventional WWTPs, activated sludge process-based treatment is the most widely utilized technique for antibiotic removal. Their treatment efficiencies vary considerably due to the different physical and chemical properties of different types of antibiotics, as well as variation in operating conditions of different WWTPs (Michael et al., 2013). Yu et al., 2009 reported that the removal of sulfamethoxazole (SMX) was 65–96% in a WWTP in China when the influent SMX concentration was 0.5–10 μg/L. However, Brown et al., 2006 and Ternes et al., 2007 only observed 20–24% SMX removal from municipal wastewater. Recently, higher antibiotic removal was reported with treatment technologies such as advanced oxidation processes (AOPs) with a principle of Fenton reaction [reaction (1)], and especially the photo-Fenton

process, electro-Fenton process, and heterogeneous magnetic metal catalytic Fenton process (Gonzalez et al., 2007; Pang et al., 2015; Wang & Bai, 2017; Zhang et al., 2016). The hydroxyl radicals (·OHs) produced by the Fenton reaction are highly effective against a variety of refractory organic pollutants including pharmaceuticals, pesticides, and dyes. However, the unsustainable issues with these AOPs are the comparatively high energy consumption and cost of operation and maintenance, due to UV radiation, ozone production, or reagents (e.g., hydrogen peroxide) injections, limiting their applications (Ahmed et al., 2017).

Attempts have been made to address these inadequacies by using heterogeneous iron catalysts in the Fenton reaction, and some recent investigations have suggested that magnetite might be a potential Fenton catalyst in a neutral pH environmental (T. Li et al., 2021; Liu et al., 2021; Zhao et al., 2021). Magnetite, which contains Fe (II) and Fe (III), is an electrical conductor in which electrons can be transported between the two oxidation states of iron. According to previous studies, the addition of magnetite particles to activated sludge caused some unconventional phenomena. For instance, when activated sludge in a sequencing batch reactor (SBR) was exposed to magnetite nanoparticles for a prolonged period, the generated highly reactive oxidative species (hROS) and lactate dehydrogenase would cause toxicity to the microbial activity (Ma et al., 2017). However, Peng et al., 2018 and Ikemoto et al., 2001 found an increase in the number of iron-reducing bacteria (IRB) and improved sludge hydrolysis after the addition of magnetite to anaerobic sludge. Under long-term exposure to magnetite, the efficacy of activated sludge in terms of both antibiotics and COD removal has not been researched. In addition, the continuous demand for H_2O_2 to activate the Fenton reaction is unsustainable and uneconomical. Moreover, H_2O_2 is easily decomposed into water owing to its unstable nature. Consequently, it is more appealing to generate H_2O_2 in-situ rather than transporting it to the application site for

pollutant degradation. H_2O_2 was found to be produced under aerobic conditions by IRB via microbial oxygen respiration (Han et al., 2019). It was also reported that some facultative anaerobic bacteria can utilize NADH, lactate, and pyruvate accumulated in their cells under anaerobic conditions and produce H_2O_2 after oxygen exposure (discussed in chapter 2). However, there have been few studies on microbial H_2O_2 production by mixed culture of microorganisms in activated sludge.

In our previous study (Shen et al., 2021), an anaerobic/aerobic SBR supplemented with magnetite was proposed as an advanced activated sludge process to simultaneously remove SMX and COD in wastewater under neutral conditions without an external supply of H_2O_2. Significant SMX removal was observed in the SBR with magnetite. However, these results were not obtained under steady-state operation because only four cycles (eight days) were conducted. It is expected that H_2O_2 may accumulate in a short time when the activated sludge is aerated, which may lead to the continuous production of ·OHs. The ·OHs production is considered effective for the degradation of refractory pollutants. SMX, a structural analog of para-aminobenzoic acid (PABA), the mechanisms by which it can treat human and animal infections is to prevent the production of dihydropteroic acid by blocking the dihydropteroate synthase, thereby restricting the growth of microbes (Bhattacharjee, 2022). In this study, SMX was used as a model antibiotic in this study because it is ubiquitously present in raw wastewater and it impacts aquatic organisms under various exposure situations even in trace quantities (Rodrigues Pires da Silva et al., 2021). Although the concentrations of SMX found in municipal wastewater range from a few nanograms to tens of micrograms, the concentration of SMX used in this study was set at a much greater level (1 mg/L) to evaluate the potential performance of the proposed SBRs.

The main objectives of this study were to verify the stability of long-term treatments using anaerobic/aerobic SBRs with magnetite. To achieve this, (i)

three identical SBRs with and without magnetite were constructed and run for an extended period of time to achieve steady-state conditions, (ii) the treatment performances of the SBRs at different magnetite dosages and hydraulic retention time (HRT) conditions were investigated.

3.2 Materials and experimental design

3.2.1 Batch experiment for microbial H_2O_2 production

Three experimental series (A, B and C) were carried out to investigate the microbial H_2O_2 production by using different types of inoculants activated sludge and iron catalysts. Each series contained 3 sets of experiments. Inoculant sludge and synthetic wastewater were added into centrifuge tubes with a working volume of 10 mL and the effective volume for all series was 7 mL. Table 1 shows the initial additions in terms of inoculant activated sludge, iron catalysts and COD concentrations for each series. The initial mixed liquor suspended solids (MLSS) for all series was set as 3000 mg/L (excluding iron catalyst). The experiment was operated for 7 cycles at room temperature (20-25℃) and each cycle consisted of 3-day anaerobic period and 3-day aerobic period. Before starting the anaerobic stage, all centrifuge tubes were purged with nitrogen gas for 10 min at a flow rate of 0.03 L/min to remove O_2 and then capped with the matching covers. After 3 days of anaerobic period, the tubes were opened and aerated to create an aerobic environment. At the end of each aerobic period, all tubes were centrifuged at 12,000 rpm at room temperature for 10 min. One milliliter of supernatant was withdrawn to quantify H_2O_2 production. To maintain the total effective volume of 7 mL, one milliliter of synthetic wastewater was then replenished to the tubes.

Table 1. Initial conditions of synthetic wastewater, inoculant sludge, iron catalyst and COD concentrations in the batch experiments.

	Synthetic wastewater (mL)*	Inoculant sludge	Iron catalyst		Initial COD concentration (mg/L)
			Fe_3O_4	$FeCl_3$	
A-1	3	4 mL aerobic sludge	-	-	171
A-2	3	4 mL aerobic sludge	1 g/L	-	171
A-3	3	4 mL aerobic sludge	-	0.1 M	171
B-1	6	1 mL anaerobic sludge	-	-	342
B-2	6	1 mL anaerobic sludge	1 g/L	-	342
B-3	6	1 mL anaerobic sludge	-	0.1 M	342
C-1	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	-	-	209
C-2	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	1 g/L	-	209
C-3	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	-	0.1 M	209

*In cycles 2 to 7, 1 mL of supernatant was replaced with 1mL of synthetic wastewater.

3.2.2 Long-term SBR experimental design

Figure 3 illustrates the apparatus used for the long-term SBR experiment. Three lab-scale SBRs with 2 L effective volume were established for the advanced activated sludge process experiment. They were labelled as SBR0 (no magnetite, control), SBR1 (containing 1 g/L of magnetite), and SBR2 (containing 3 g/L of magnetite). A steel stirrer and tubes for influent, effluent, nitrogen, oxygen, gas collection, and sampling were placed through the plate cover. Each reactor was fed with synthetic wastewater and inoculant sludge. The inoculant sludge comprised anaerobic and aerobic sludge in a 1:1 ratio of MLSS. SBR0, SBR1, and SBR2 were set with an initial MLSS (excluding magnetite) of 3 g/L. The whole experiment was carried out at room temperature (20–25 °C) and the initial pH of all reactors was 6.5–7.5 without adjustment. The system was purged with nitrogen at a gas flow rate of 0.5 L/min for 5-10 min at the beginning of each cycle to maintain the dissolved oxygen (DO) at 0–1 mg/L for anaerobic periods. Air was introduced via an aeration device during the entire aerobic period.

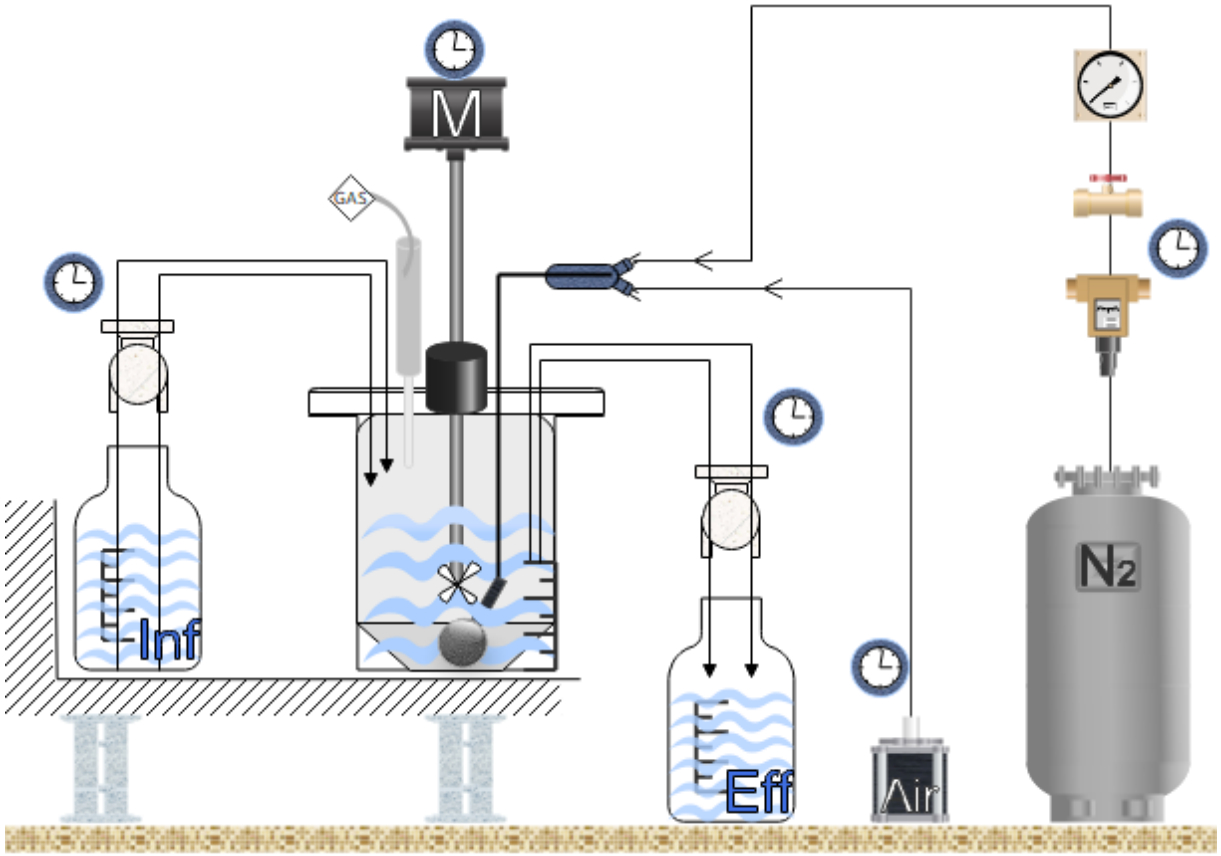

Figure 3. Diagrammatic illustration of the SBR experimental apparatus.

3.2.3 Long-term SBR experimental operation

Three SBRs have been operated in a sequence of five phases for almost two years so far. Phase I, Phase II, Phase III, Phase IV, and Phase V consisted of 19, 79, 172, 104, and 107 cycles, respectively. One cycle in the SBR was mathematically represented by Eq. (8), where the total time of one cycle (t_c) is the summation of influent, anaerobic period, aerobic period, setting, and effluent. Gradually shortened HRT was given in the experiment and was calculated using Eq. (9) (Thakur et al., 2013). The operation of one cycle (Figure 4) proceeded with an influent period and followed by an anerobic period and an aerobic period. After the reaction time, the stirrer and gas supply were stopped for 30 min to enable sludge settling, then the supernatant was replaced with new synthetic wastewater with a volume exchange ratio of 50%. Samples were collected at predetermined time intervals.

$$t_c = t_{INF} + t_{AN} + t_{AE} + t_S + t_{EFF} \quad (8)$$

Where, t_{INF} is the influent time, t_{AN} is the time of anaerobic period, t_{AE} is the time of aerobic period, t_S is the settling time, t_{EFF} is the effluent time.

$$HRT = \frac{(t_c)}{V_{ex}/V_t} \quad (9)$$

Where, V_{ex} is the volume of exchanged wastewater (1 L in this study), V_t is the total working volume (2 L in this study).

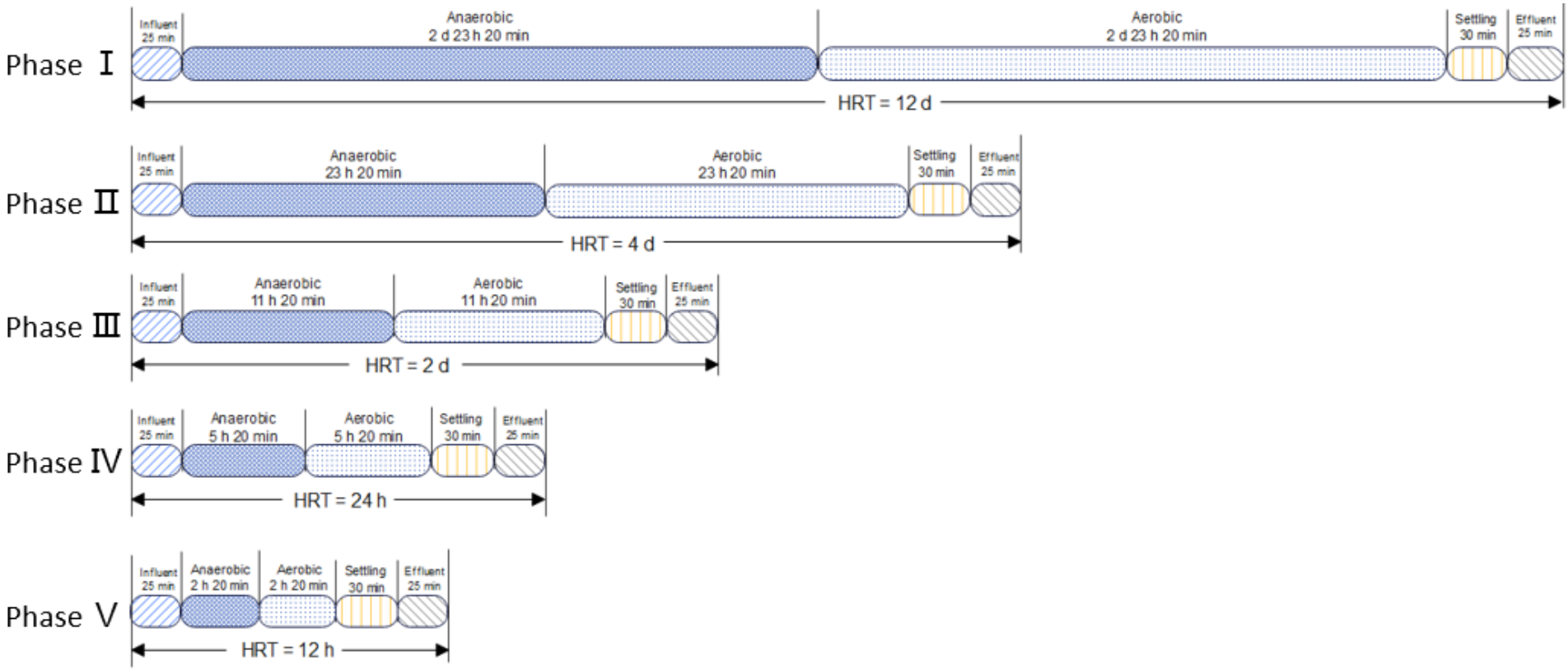

Figure 4. The outline of different periods included in one cycle during different phases.

3.2.4 Chemicals

Aerobic activated sludge was collected from a return activated sludge of a WWTP in the Kanto Region of Japan, while anaerobic sludge was provided by Prof. Li Yu-You at Tohoku University, Japan. These seed sludges were different from those used in our previous study (Shen et al., 2021) to verify the reproducibility of the experiments. SMX, micron-sized magnetite powder (< 5 μm), 3-(dimethylamino) benzoic acid (DMAB), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) were purchased from Sigma-Aldrich (Darmstadt, Germany). SMX stock solution was prepared by dissolving 200 mg SMX into 10 mL pure methanol (98%). 1 mg/L of SMX solution was made by diluting the SMX stock solution before each replacement of synthetic wastewater. Horseradish peroxidase was purchased from Fujifilm Wako (Osaka, Japan). 1,10-phenanthroline hydrochloride monohydrate and hydroxylamine hydrochloride were purchased from TCI (Tokyo, Japan). The composition of synthetic wastewater was determined by referring to the literature (Sudo, 1993), and the concentration was adjusted to provide an influent COD of 405 mg/L: casein peptone 150, glucose 150, sodium acetate 52, urea 52, $CaCl_2 \cdot 2H_2O$ 4, $MgSO_4 \cdot 7H_2O$ 2, NaCl 6, KH_2PO_4 88 and SMX 1 mg/L. All reagents were of reagent grade and were used without further purification. All solutions were prepared using deionized water from a Millipore system.

3.2.5 Analytical procedures

3.2.5.1 Water quality parameters analysis

Parameters including MLSS, mixed liquor volatile suspended solids (MLVSS), and sludge volume index (SVI) were measured according to Standard Methods (2005). The COD analysis was performed using the closed reflux method in accordance with Standard Methods (2005) (Federation & Association, 2005). DO and pH were measured using an Orion Star™ A223 DO meter and an EutechTM PC 450 digital meter, respectively (Thermo Fisher Scientific, Waltham, MA). The COD and SMX removal efficiencies were calculated using Eq. (10).

$$Removal\ efficiecy = \frac{C_{inf} - C_{eff}}{C_{inf}} \times 100\% \quad (10)$$

where C_{inf} and C_{eff} indicate the influent and effluent concentrations of each cycle, respectively.

3.2.5.2 SMX

An Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) equipped with a diode array detector was used to measure the concentrations of SMX. The detection wavelength and column temperature were set at 268 nm and 40 °C, respectively. Before analysis, samples were centrifuged at 10,000 rpm for 5 min at room temperature (20–25 °C) and the supernatant was then filtered through a 0.45 μm syringe filter (PTFE025045L, Membrane Solutions). A sample volume of 20 μL was injected using an autosampler. Separations were performed on a 4.6 x 150 mm L-column ODS (GL, Sciences, Tokyo, Japan). Mobile phase eluent A (0.3% acetic acid in acetonitrile) and B (0.3% acetic acid in ultrapure water) were delivered at a flow rate of 1.0 mL/min through the

column in time-dependent gradient proportions for 13 min. Elution was initiated with 34% A for 6 min, then increased to 100% A in 0.1 min, and maintained for 2.9 min. Then, it was returned to the initial condition in 0.1 min, and was maintained for 3.9 min. The retention time of SMX was 4 min. The standard curves obtained from calibration always had a coefficient of determination greater than 0.99.

3.2.5.3 H_2O_2

H_2O_2 concentration was quantified using a modified chromogenic assay based on the oxidative coupling of DMAB and MBTH (Ngo & Lenhoff, 1980; Okuda et al., 1991). An aliquot of 400 μL of 12.5 mM DMAB reagent (51.6 mg of DMAB into 25 mL of 0.375 M sodium phosphate buffer solution) and 80 μL of 1.3 mM MBTH followed by 20 μL of 5 units horseradish peroxidase were added to a 1 mL of sample. The reaction mixture was rested at room temperature for 5 min before being cooled at 0 °C for 10 min to terminate the enzyme activity. Before a UV-Vis spectrophotometer (UV-160 Shimadzu) was employed to collect triplicate absorption values at 590 nm, the reaction mixture was centrifuged at 10,000 rpm at room temperature for 10 min.

3.2.5.4 Fe

A modified extraction of Fe from magnetite was conducted based on a previous study (Porsch & Kappler, 2011). The presence of Fe in magnetite was identified as HCl-extractable Fe by dissolving a mixture of sludge and magnetite in 6 M HCl and was heated for 1 hour at a temperature of 150 ℃. The concentrations of dissolved ferrous ions in the HCl extraction were then measured spectrophotometrically using a modified 1,10-phenanthroline method (Stucki & Anderson, 1981; Zhang et al., 2016). The total Fe

concentration was determined by reduction of ferric to ferrous ions using hydroxylamine hydrochloride. The difference between ferrous and total Fe was used to calculate the ferric concentration. Prior to analysis, all samples were filtered through a 0.45 μm syringe filter (PTFE025045L, Membrane Solutions). X-ray diffraction (XRD) was performed using an X-ray diffractometer (INET-Ultima III Rigaku) with a Cu Kα radiation source.

3.3 Results and Discussions

3.3.1 Microbial H_2O_2 production in batch experiments

During the whole experiment, control groups (A-1, B-1 and C-1) and experimental groups with magnetite (A-2, B-2 and C-2) were maintained at circumneutral pH (6.50-7.40) without adjustment. On the other hand, the pH of experimental groups with $FeCl_3$ (A-3, B-3 and C-3) was in the range of 2.48-4.89. As shown in Figure 5, H_2O_2 produced in series A (aerobic sludge) was remarkably less than that in series B (anaerobic sludge) and C (mixture of aerobic and anaerobic sludge). In series B and C, the concentrations of H_2O_2 in control groups (B-1 and C-1 with no iron catalysts) were lower than those in experimental groups (B-2, B-3, C-2 and C-3). Anaerobic sludge and mixed sludge containing $FeCl_3$ (B-3 and C-3) produced more H_2O_2 compared with other groups during the first half of the experiment, while H_2O_2 concentrations decreased from the 3rd cycle to the end of the experiment. On the other hand, it is interesting to note that the concentration of H_2O_2 after aerobic periods in the groups with the addition of magnetite (B-2 and C-2) increased gradually in both anaerobic and mixed sludge and reached the peak concentrations of 15.0 and 16.4 μM, respectively. In B-2 and C-2, as magnetite consists of undissolved Fe (III) and Fe (II), structural Fe (III) in magnetite might be reduced by IRB to insoluble Fe (II), which was expected to enhance the production of H_2O_2 on the

surface of magnetite particles (Cheng et al., 2016; Voinov et al., 2011). The redox of insoluble magnetite prevented the loss of iron even under neutral conditions. Furthermore, it was considered that IRB were enriched in the existence of magnetite particles during anaerobic periods, resulting in the increasing amount of H_2O_2 production via reaction (3), (4), (5), and (6). Due to the stable and promising H_2O_2 production under neutral pH in the groups with magnetite, it was applied as the catalyst in the subsequent sequencing batch experiment.

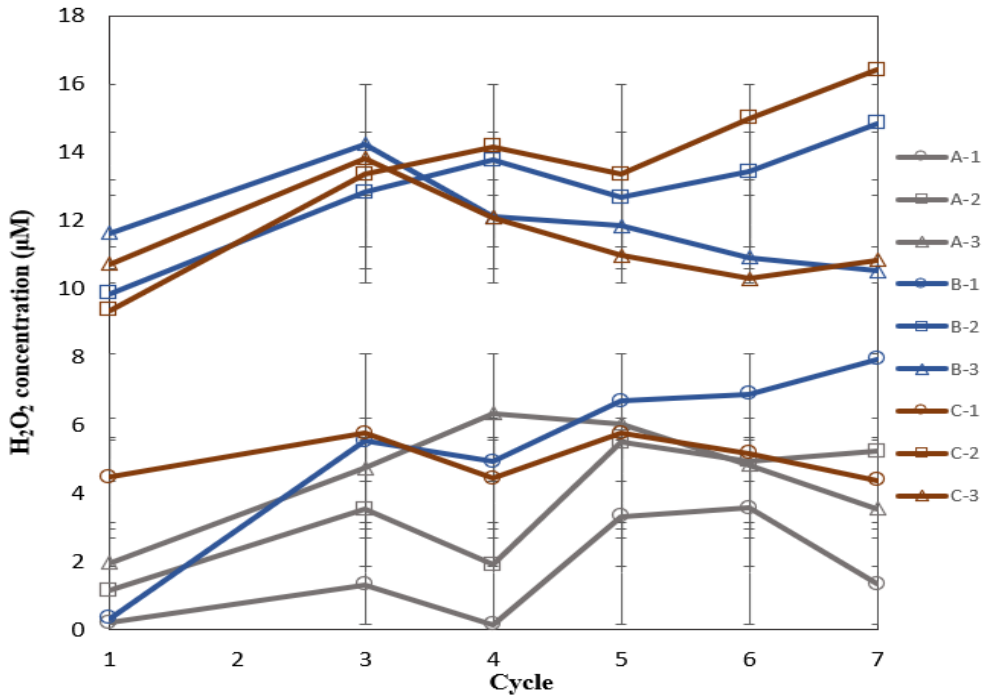

Figure 5. Microbial H_2O_2 generation after aerobic period under different sludges and iron catalysts.

3.3.2 Advanced activated sludge process

3.3.2 1 Sludge physical properties

pH and DO are crucial for biological processes since both aerobic and anaerobic conversions are pH and DO dependent. The whole anaerobic

process proceeds at its fastest pace in the pH range 6 to 8. Below a pH of 6, the activity of anaerobic microbes such as methane-forming bacteria declines drastically. For aerobic process, the nitrification process also relies on pH, with an ideal pH range in the 8-9. Typically, low pH levels are often the culprits behind issues in biological treatment processes. Moreover, compared to heterotrophic bacteria, certain homogenous bacteria, such as nitrifying bacteria, are more sensitive to low oxygen concentrations. Nevertheless, nitrification may occur at high oxygen concentrations, such as in an oxygen-only plant. This process is even not inhibited by 60 g oxygen/m^3. It is important to control the DO levels during anaerobic periods since oxygen limits the denitrification process (Henze et al., 1995).

Figures 6 and 7 show the temporal variations of pH and DO levels, respectively. The pH was slightly fluctuated during Phases I and II before becoming stable and remaining between 7.0 and 8.5 under both anaerobic and aerobic conditions. These comparable pH readings in three SBRs suggested that the pH maintenance was not significantly impacted by the addition of magnetite. Based on Figure 7, which depicts the temporal change of DO, it could be inferred that, DO throughout the anaerobic phases was maintained blow 1.0 mg/L, with the exception of several cycles in Phases I and II. The lowered DO during the later phases was because (i) the increased nitrogen input at the start of each anaerobic period; (ii) the increasing number of microorganisms (see the MLVSS in Figure 8) led to a faster oxygen consumption rate. Furthermore, there were considerable fluctuations in DO values during the aerobic periods, and all three SBR systems showed a declining tendency. Air was supplied throughout each whole aerobic period at a predetermined aeration rate; however, the surface of the diffusor stone was easily attached with the sludge flocs which hindered the aeration volume and resulted in vertiginous DO values. The lower aerobic DO in Phase V was mostly

due to the shorter HRT that provided less oxygen. These observed results suggested that consideration needs to be given to the management of DO.

MLSS, MLVSS, and SVI measurements were conducted to assess the physical and biological sludge characteristics, which were measured at predetermined time intervals (Figure 8). Generally, MLSS and MLVSS concentrations of all reactors grew slightly as the experiment progressed. Throughout the experiment, the MLSS of SBR0, SBR1, and SBR2 increased from 3.0, 3.9, and 5.9 g/L to 14, 12, and 11 g/L, respectively, while the MLVSS of SBR0, SBR1, and SBR2 increased from 2.1, 2.3, and 2.4 g/L to 11.4, 9.0, and 8.6 g/L, respectively. According to these observations, the activated sludge and microorganisms adapted to their environments and were acclimated and enriched throughout the long-term operation of all the systems. The SVI of the three SBRs showed a slight downward trend in Phases I and II and a sharp decline trend in Phases III and IV before maintaining at approximately 35, 43, and 44 mL/g MLSS in SBR0, SBR1, and SBR2, respectively, in Phase V. SVI has been considered the standard measure for determining the physical properties of activated sludge in terms of settling. A low SVI implies good sludge compaction, while a higher SVI generally indicates sludge bulking (Ng et al., 1993). In the first four phases, the SVI values of SBR1 and SBR2 were lower compared to those of the SBR0. Namal, 2019 reported that adding magnetite to anaerobic reactors could result in a better-quality effluent owing to the enhanced settleability of the sludge. However, compared to SBR1 and SBR2, SBR0 exhibited lower SVI in Phase V and greater MLSS and MLVSS in the last three phases. It is suspected that this is because bacteria consumed organic matter to synthesize polymers. Microorganisms can produce biopolymers either directly through fermentation or chemically through the polymerization of monomers by processive enzymes. For example, methanol is one of the carbon sources as the fermentation substrates, and monomers can be sugars, fatty acids, and nucleic acids (Mohan et al., 2016; Sukan et al., 2015). It was reported

that both anaerobic and aerobic activated sludges can yield biopolymers. Protein predominates over all other fractions in anaerobic sludge polymers, whereas glucose predominates in aerobic activated sludge (Morgan et al., 1990). While magnetite quite likely to some extent affected the ability of microbes to synthesize biopolymers. Further investigations are required to identify the creation of polymers, and this phenomenon can be explained more convincingly by determining the amount of polymers extracted from activated sludge.

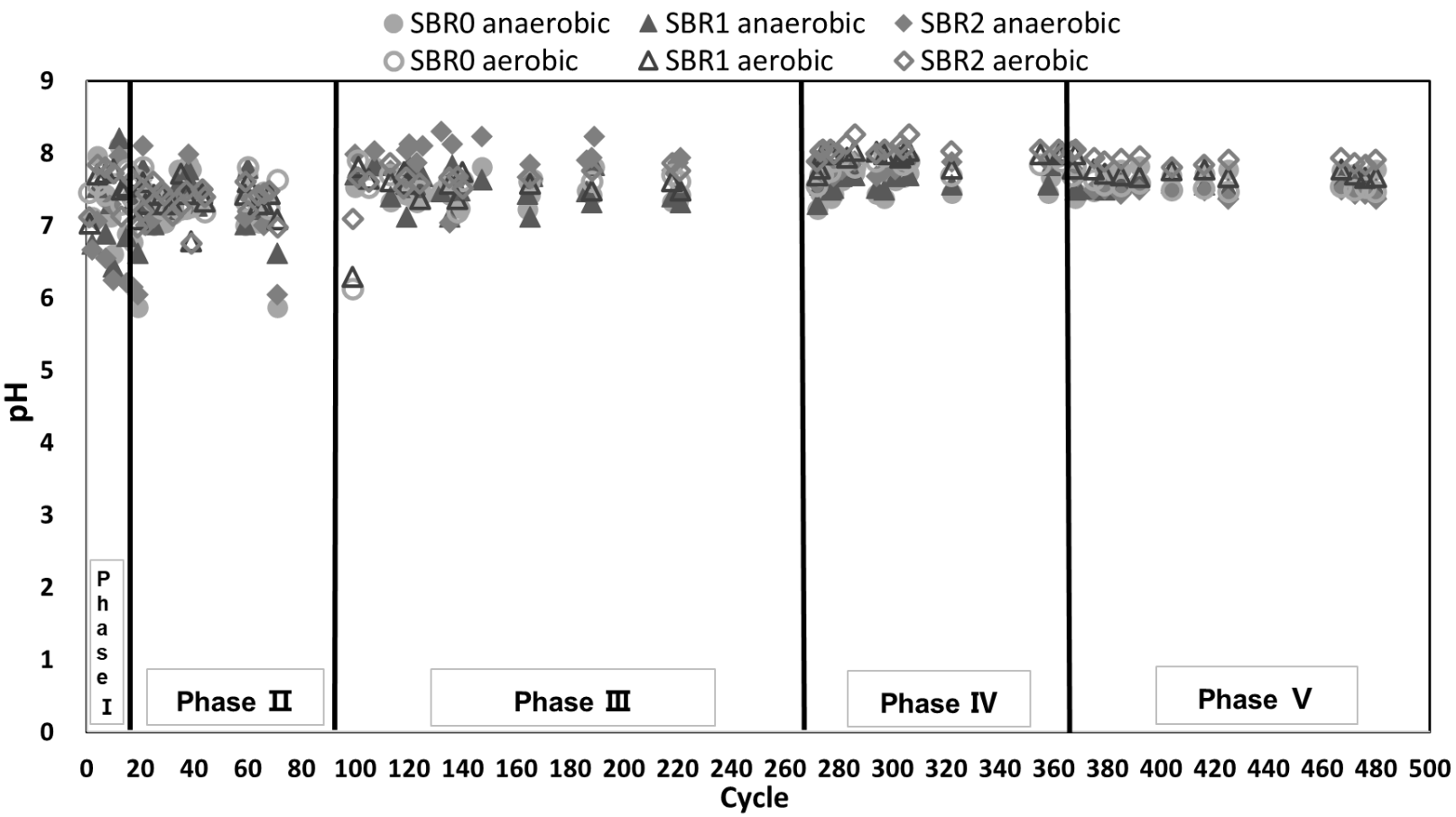

Figure 6. Profiles of temporal variations of pH in both anaerobic and aerobic periods.

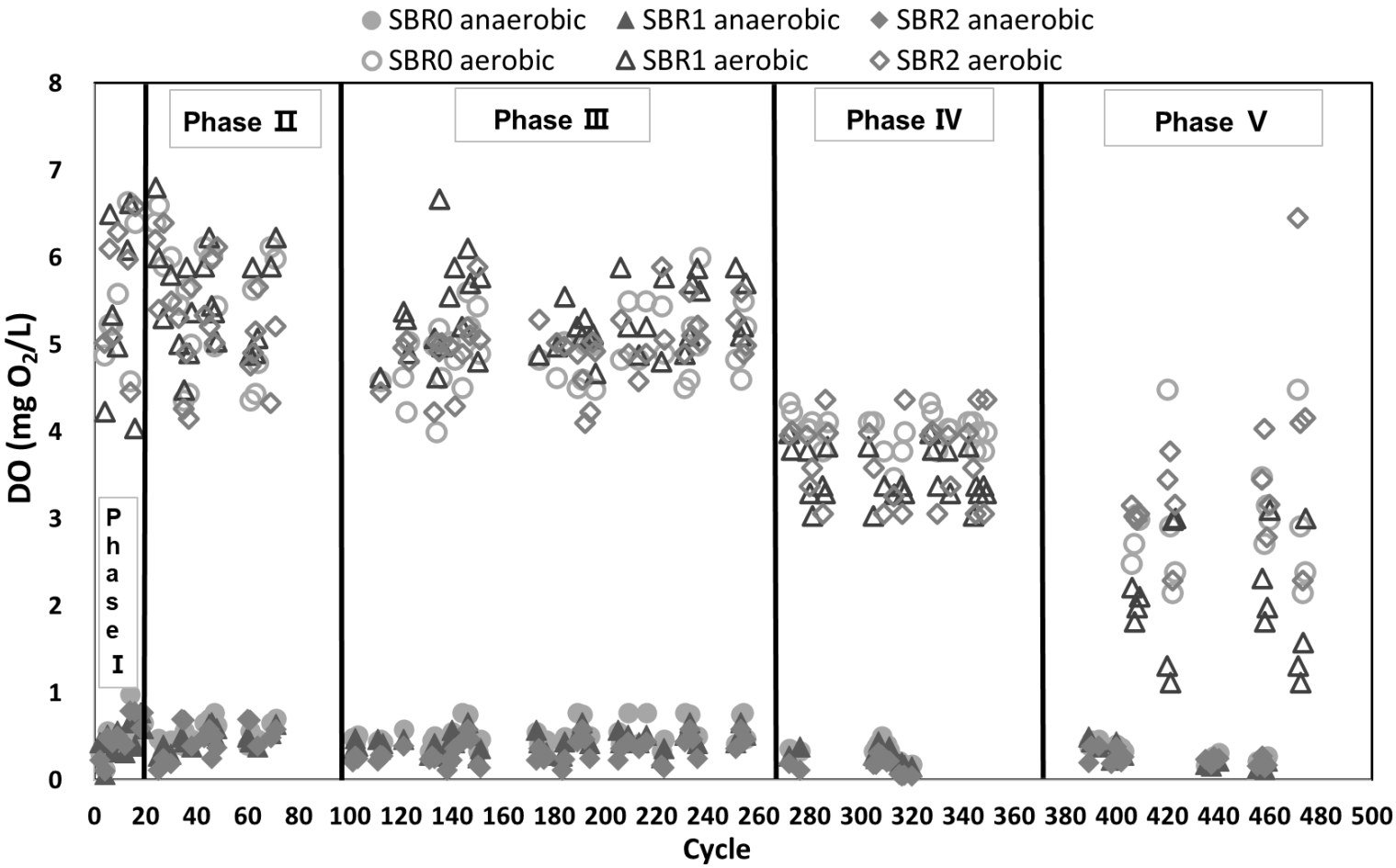

Figure 7. Profiles of temporal variations of DO in both anaerobic and aerobic periods.

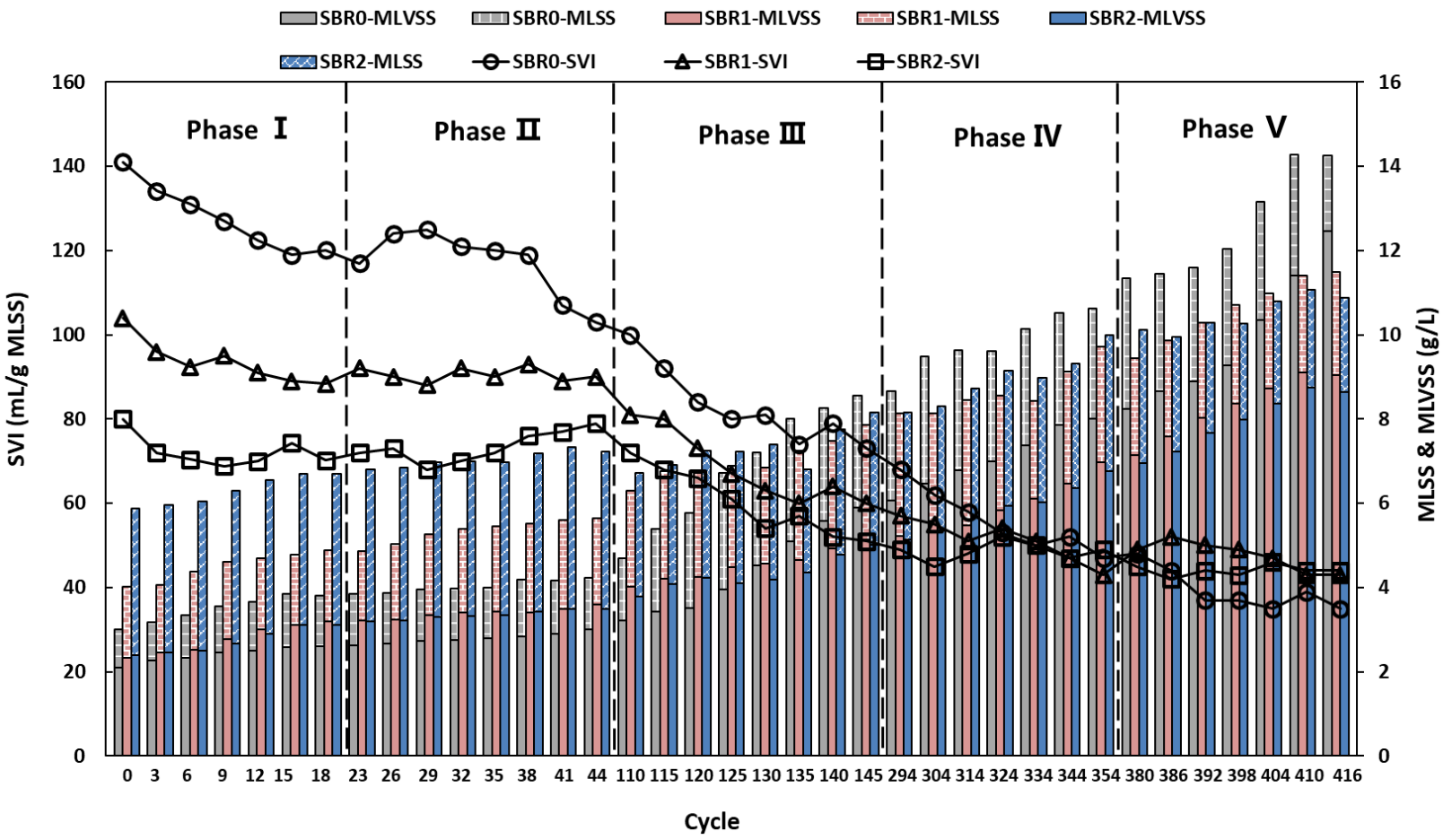

Figure 8. Profiles of MLSS, MLVSS, and SVI values measured at predetermined time intervals. Cycle “0” indicates the initial values of the inoculant sludge.

3.3.2.2 Removal of COD

COD removal is an important criterion for determining the performance of conventional wastewater treatments. The changes in COD removal are shown in Figure 9. In SBR0 and SBR1, COD removal increased and eventually approached steady-state levels in Phase I, while those in the other phases were almost maintained at steady-state levels. SBR1 and SBR2 had relatively similar amount of COD removal in Phases I and II, which was about 6% higher than that of SBR0; however, SBR2 showed a declined removal efficiency from 87% to 82% in Phase III and subsequently rose to around 89% in Phases IV and V. Throughout the experiment, SBR1 kept having the highest COD removal efficiency at around 93% under steady conditions. This shows that the overall COD removal was not significantly affected when the anaerobic and aerobic period was shortened from three days to three hours. When 1 g/L of magnetite was added to the SBR, the COD removal was enhanced by approximately 6% when HRT was 12, 4, and 2 days, and by approximately 3% when HRT was 1 day in comparison to the control SBR (SBR0). The results further imply that the biodegradation of conventional organic pollutants in the Bio-Fenton SBR was not substantially affected by the magnetite dosage since no significant differences in COD removal efficiency were observed between SBR1 and SBR2. Yang et al., 2019 also proved that the addition of magnetite particles to microaerobic up-flow sludge reactors did not inhibit microbial growth and organic decomposition reactions due to the lower toxicity of micron-scale particles compared with that of nanoscale particles.

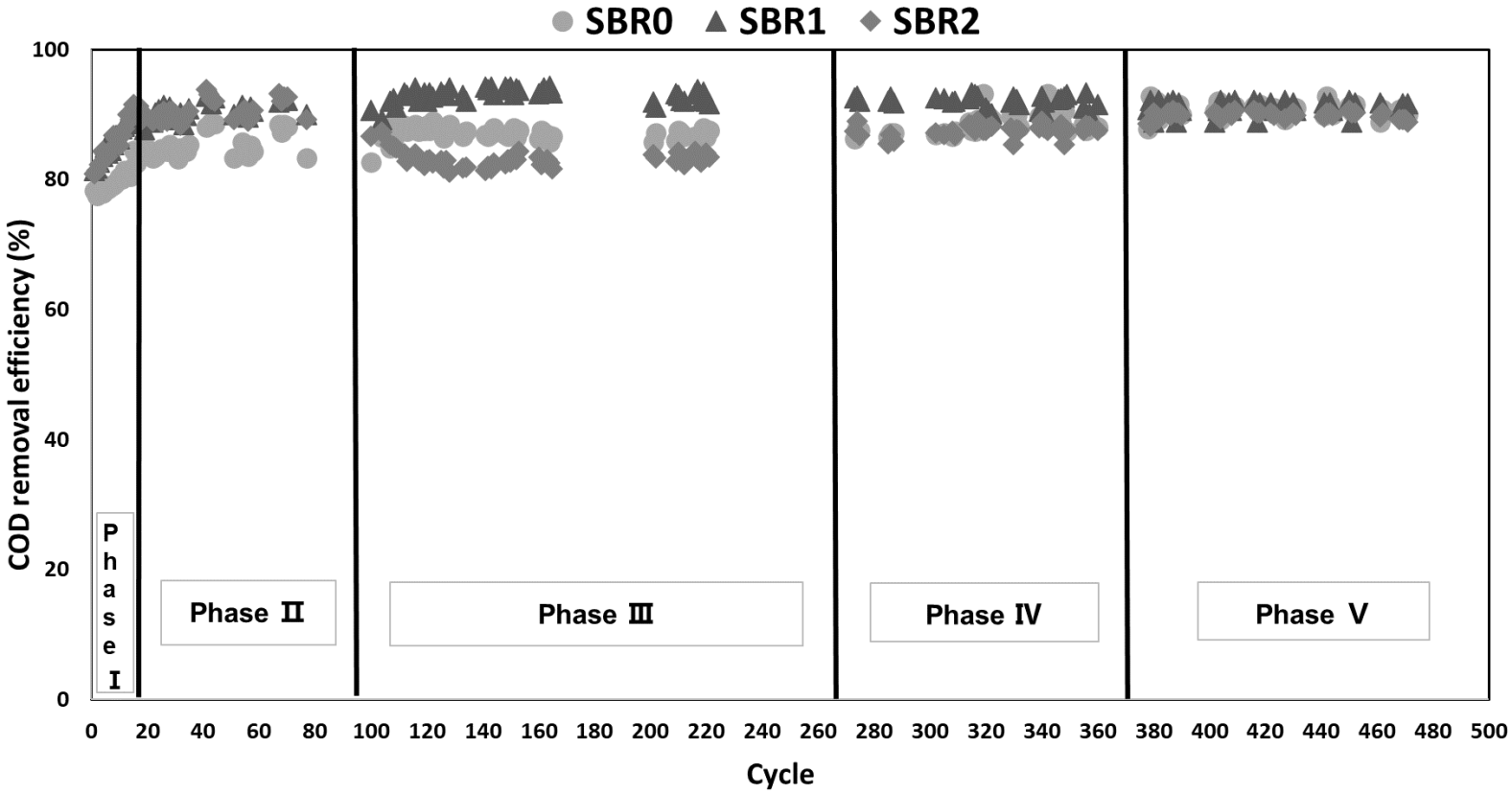

Figure 9.Overall COD removal efficiency in SBR0, SBR1, and SBR2 observed at predetermined time intervals.

3.3.2.3 Removal of SMX

Figure 10 displays the overall SMX removal efficiency in the three SBRs, with patterns similar to those of the H_2O_2 concentrations (Figure 11). In terms of SMX removal, SBR0 and SBR1 had comparable tendencies. During Phase I, they exhibited upward and asymptotic trends during, and remained stable at roughly 48 and 88% during Phase II, and then marginally increased to around 58% and 100% in Phase III, respectively. Afterwards, in Phase IV, SBR1 continued to demonstrate 100% SMX removal whereas SBR0 showed a drop from 58% to 50%. As was the case with SBR1, SBR2 outperformed SBR0 in Phases I and II before sharply declining to approximately 60% and 50%, respectively, in Phases III and IV. When HRT was shortened to 12 h (Phase V), similar lower SMX removal efficiencies in the range of 21 to 26% were observed in the three SBRs.

When the magnetite dose was 1 g/L, SMX removal efficiency rose slightly and eventually reached to complete elimination when the anaerobic and aerobic periods were reduced from 3 days to 6 hours. On comparing the efficiency of SBR1 and SBR2 during Phases III and IV, significant differences were noticed; this may be due to the reduced generation of H_2O_2 in SBR2 (Figure 11), suggesting that the greater concentration of magnetite under shorter HRT circumstances impacted microbial activity. The dramatically reduced removal efficiency in SBR1 during Phase V was because of the low DO under aerobic conditions (Figure 7), which might limit the microbial production of H_2O_2 (Figure 11). The removal of SMX was not improved when the magnetite dosage was 3 g/L, except for in Phases I and II. This phenomenon may occur because excess magnetite inhibited the oxygen transfer rate, thereby affecting bacteria's conversion of oxygen to hydrogen

peroxide. Future research is required to determine the dissolved oxygen concentration within the bacteria.

SMX concentrations after anaerobic periods were also monitored (Figure S1), and the results demonstrated that SMX was primarily removed in aerobic phases because less than 0.13 mg/L of SMX was decreased during anaerobic periods. In section 3.3.2.4, the microbial H_2O_2 production is discussed, and it was observed that H_2O_2 was mainly produced during aerobic periods. It can be considered that the oxidation of H_2O_2 was catalyzed by the Fe (II) existed in the magnetite, generating hydroxyl radicals which decomposed SMX. Both biodegradation and sorption contributed to SMX removal in the biological treatment. However, sorption to activated sludge was determined to be a minor removal mechanism for SMX as previous studies have demonstrated that solid sorption was considerable in refractory organic pollutants with an n-octanol-water partitioning coefficient (log K_{OW}) above 4.0, while SMX has a log K_{OW} value of 0.89 (Brillas et al., 2009). Our preliminary batch experiment showed that the maximum adsorption rate of SMX was 8.3%, indicating that less than 0.1 mg/L of SMX was adsorbed onto magnetite (Figure S2). SMX biodegradation via activated sludge process ranged from almost complete removal to very limited biodegradation. Our results obtained from SBR0 were similar to those reported in previous literature (Collado et al., 2013), where limited SMX removal was observed in conventional activated sludge systems with a removal efficiency of 36.5 ± 11.5% when the influent SMX concentration was 66.8 ± 7.0 μg/L. In contrast, Suarez et al., 2012 observed that anoxic and aerobic activated sludge systems reached SMX removal efficiencies of approximately 64–70%. Many factors can affect the biological treatment, such as water components and microorganism species (Wang & Wang, 2018). The presence of magnetite caused a change in the types of microorganisms and corresponding biological reactions, which also affected the treatment performance in removing SMX (O'Flynn et al., 2021). The predominant

microorganism species need to be identified in further studies to evaluate the performance precisely.

3.3.2.4 Microbial production of H_2O_2

After aerobic period, the H_2O_2 concentration in the bulk phase was quantified at predetermined time intervals (Figure 11), and the accumulated H_2O_2 concentration was dependent on the amount of H_2O_2 released into the system as well as the balance between its production and consumption. During Phases Ⅰ and Ⅱ, three SBRs exhibited a rise and subsequent asymptotic approach to a certain level of H_2O_2 concentrations. For example, H_2O_2 concentrations in SBR1 and SBR2 climbed from approximately 15 to 40 μM in Phase Ⅰ and from 40 to 51 μM in Phase Ⅱ. H_2O_2 concentration in SBR0 increased from 11 to approximately 20 μM in Phase Ⅰ and remained stable at about 22 μM in Phase Ⅱ.

It was interesting to find a rapid decline in H_2O_2 concentration in all reactors when HRT was reduced from 4 days (Phase Ⅱ) to 2 days (Phase Ⅲ), and almost no H_2O_2 was detected under the HRT of 12 h (Phase Ⅴ). It was supposed that SMX was decomposed by ·OHs produced by the Bio-Fenton reaction. Despite this, SMX was removed completely by SBR1 even during Phases Ⅳ where H_2O_2 concentration in liquid phase was below 15 μM. The H_2O_2 concentration in sludge flocs was then measured and the results are shown in Figure 12. Quite high H_2O_2 concentration (60 - 70 μM) in sludge flocs withdrawn from SBR1 during Phase Ⅳ was observed and this explained the 100% removal of SMX (Figure 10). In Phase Ⅴ, H_2O_2 concentration was small in both liquid phase and sludge flocs, which resulted in a reduction in the SMX removal efficiency (Figure 10). These findings revealed that the microbial H_2O_2 production was affected when HRT was reduced. Moreover, as was covered in Chapter 2, H_2O_2 is mainly produced via microbial respiration when microbes are presented with

oxygen. The lower DO concentrations during aerobic periods in Phase Ⅴ (Figure 7) might also contribute to a reduction in the amount of H_2O_2 that was produced by some facultative anaerobic microbes.

H_2O_2 concentrations after anaerobic periods were below 8 μM in all three SBRs (data not shown). These results indicated that H_2O_2 was mainly produced during the aerobic phases, and the addition of magnetite enhanced its production. It was reported that *S. onedensis* MR-1 generated approximately 20–26 μM H_2O_2 under aerobic and anoxic/oxic conditions (Sekar & DiChristina, 2014). Our results for SBR1 and SBR2 showed that more than twice H_2O_2 accumulation was detected compared to that reported previously. Abiotic H_2O_2 production was investigated by conducting a preliminary batch experiment under the same operating conditions as the SBR experiment. The results showed that the abiotic production of H_2O_2 was smaller than 8 μM in the presence of 1 and 3 g/L magnetite. This indicates the accumulated H_2O_2 in SBRs was mainly microbially produced.

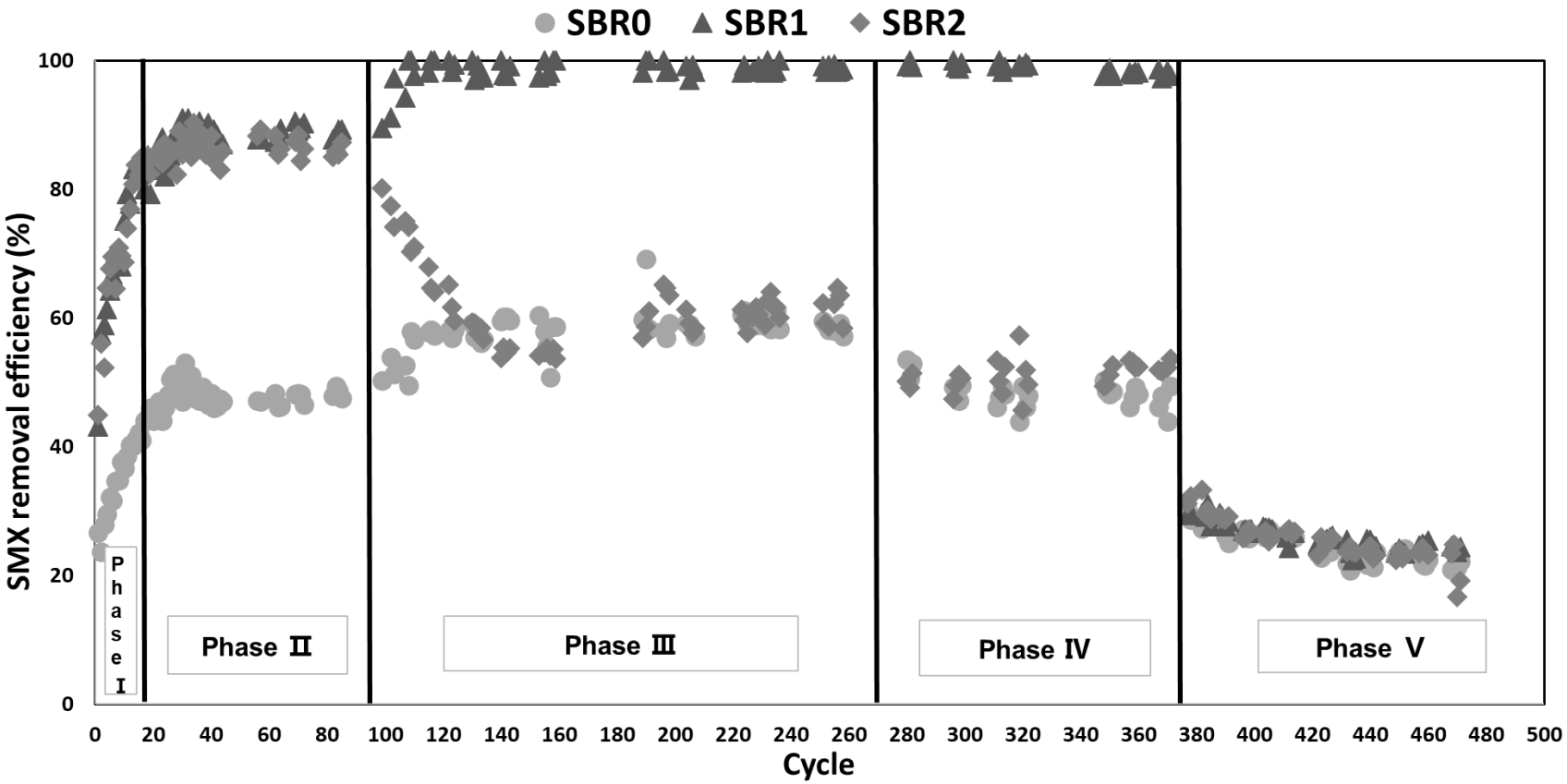

Figure 10. Overall SMX removal efficiency in SBR0, SBR1, and SBR2 observed at predetermined time intervals.

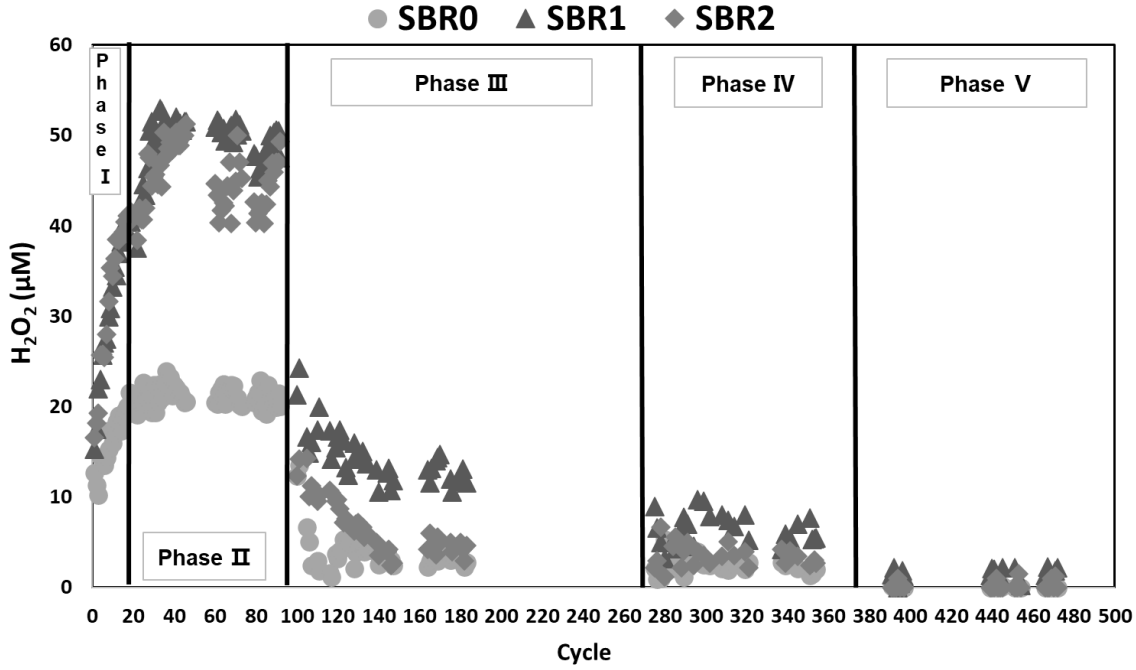

Figure 11. Observed H_2O_2 concentration in bulk phase at the end of certain aerobic periods.

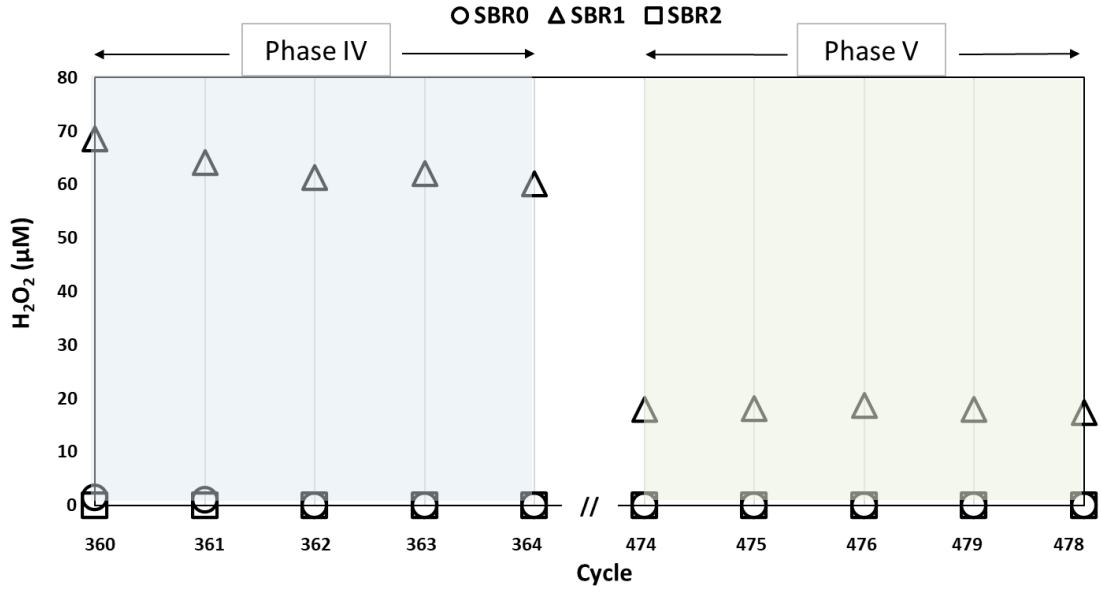

Figure 12. Observed H_2O_2 concentration in the sludge flocs under certain aerobic periods in Phases IV and V.

3.3.2.5 Redox cycle of magnetite

The reduction and oxidation of magnetite were investigated for specific cycles in each Phases. Originally, magnetite consists of Fe (II) and Fe (III) in a molar ratio of 1:2. Under the microbial metabolism effect of ferrous oxidizing bacteria and ferric reducing bacteria, magnetite can be used as an electron donor and acceptor under aerobic and anaerobic conditions, respectively. These iron-metabolizing microorganisms also have the ability to grow during the dissimilatory iron redox process (Blair et al., 2015; Mejia et al., 2016). Therefore, it can be considered that the ratio of Fe (II) and Fe (III) in magnetite changes with the participation of different bacteria. Figure 13 shows that Fe (III) concentrations decreased and increased under anaerobic and aerobic conditions, respectively, and Fe (II) concentrations exhibited the opposite trend. In SBR1 and SBR2, the amount of microbially produced Fe (II) was similar. This indicates that a large amount of magnetite was not utilized by IRB in SBR2. The total Fe concentration in SBR0 decreased from 0.04 to 0.00 mM, [Figure 13(a)], suggesting the occurrence of iron leaching. Almost negligible iron leaching was observed in SBR1 and SBR2 during the first three phases because their total Fe concentrations were maintained within a certain range [Figures 13(b) and 13(c)]. However, when HRT was lowered to 12 hours, their total Fe concentrations fell from 13 to 10 mM, and from 29 to 26 mM, respectively, associated with a decrease in the amount of anaerobic reduced Fe (III) and aerobic oxidized Fe (II). These results indicated that shorter HRT limited the potential of the redox cycle of magnetite. The solids content in the reactor was extremely high and caused uneven stirring as a result of the fast-growing of MLSS and MLVSS (Figure 8). The reliability of the sampling was negatively impacted when some of the sludge flocs sank to the bottom of the reactor when withdrawing the sample. This might also cause the decrease in total iron concentration observed in the latter two phases. It is strongly suggested that

the system be brought to a condition of entire mixing by either removing excess sludge or increasing the strength of the stirring.

During the first three phases, in SBR1 and SBR2, the reduction of Fe (III) during the anaerobic period was almost equivalent to the amount of oxidized Fe (II) during aerobic periods. This indicates that the continuous use of magnetite for the biological Fenton reaction was with great potential. Lovley & Phillips, 1986 elucidated the potential of Fe (III) reduction to utilize the reducing equivalent and contribute to organic matter decomposition. The stoichiometric relationship between microbial Fe (II) production and COD oxidation was then calculated as Δ COD/Δ Fe (II) = 6.72 mg COD/mmol Fe (II) (Henze et al., 1995; Lovley & Phillips, 1986). These results explain the approximately 6% higher COD removal in SBR1 and SBR2 than that in SBR0 during Phases I, II, and III (Figure 9). The magnetite and sludge complexes obtained from SBR1 and SBR2 after the 25th cycle (in Phase II) was subjected to XRD analysis to further elucidate the structural integrity of magnetite (Figure 14). The characteristic peaks of these two samples are consistent with the standard card of magnetite (JCPDS Card No. 00-011-0614). This suggests that maximum magnetite was not converted into other types of iron oxides during the experimental operation.

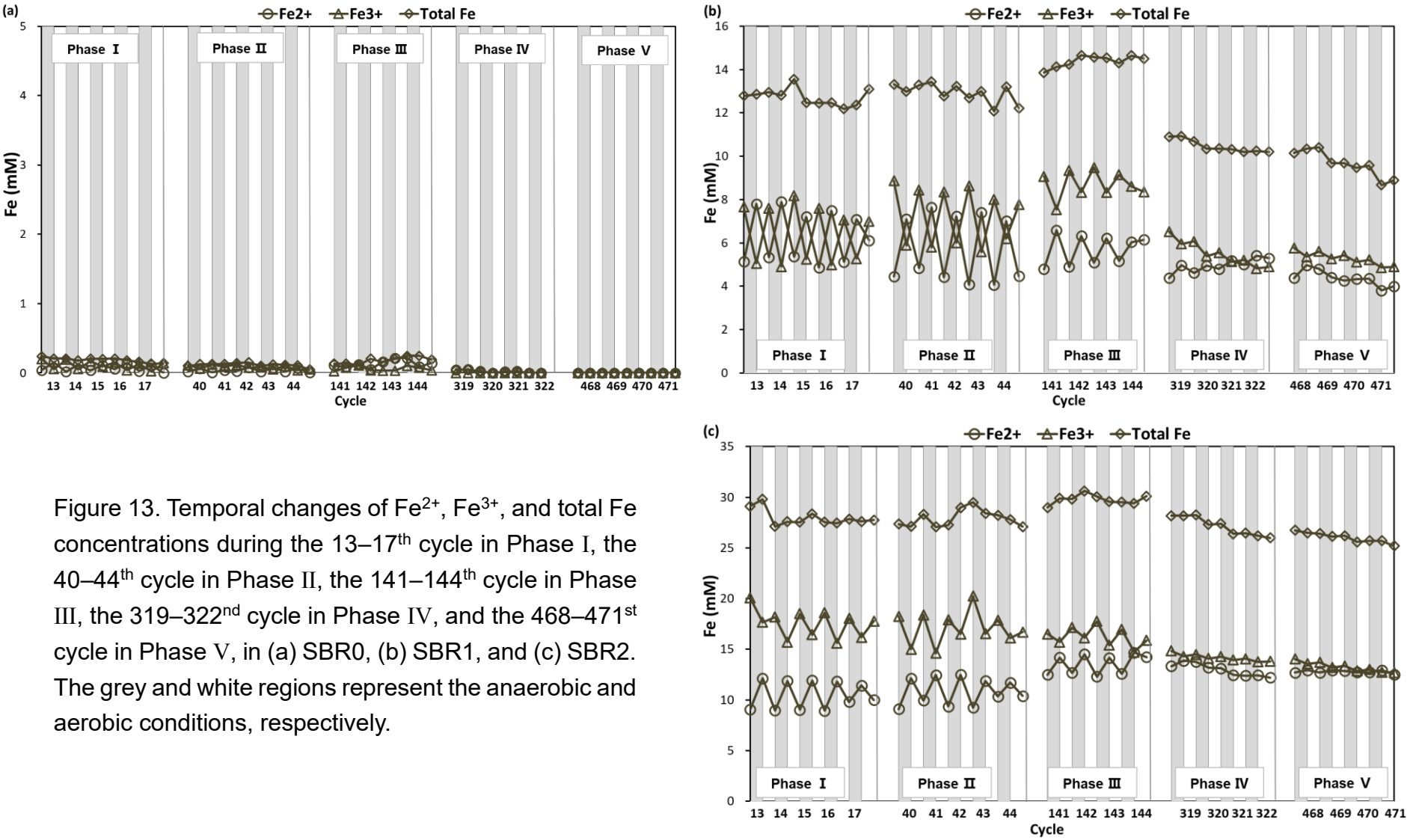

Figure 13. Temporal changes of Fe^{2+}, Fe^{3+}, and total Fe concentrations during the 13–17th cycle in Phase Ⅰ, the 40–44th cycle in Phase Ⅱ, the 141–144th cycle in Phase Ⅲ, the 319–322nd cycle in Phase Ⅳ, and the 468–471st cycle in Phase Ⅴ, in (a) SBR0, (b) SBR1, and (c) SBR2. The grey and white regions represent the anaerobic and aerobic conditions, respectively.

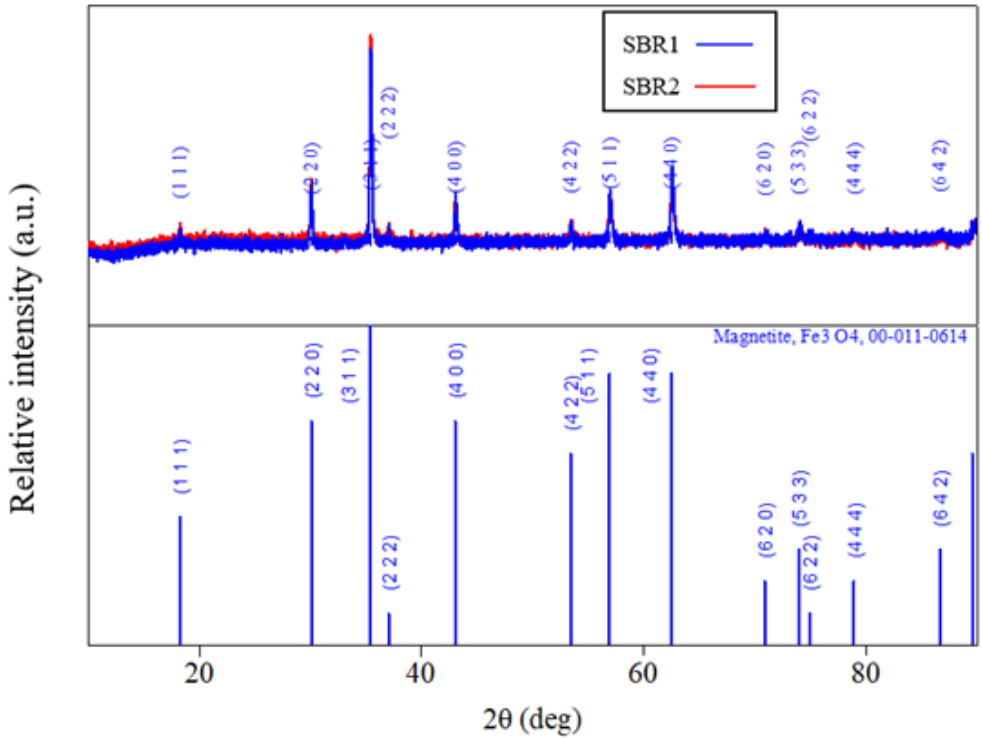

Figure 14. XRD micrographs of the magnetite and activated sludge complexes in SBR1 and SBR2 during Phase II.

3.4 Conclusions

Experiments were conducted to investigate the removal performances of the antibiotic (SMX) and COD using an anaerobic/aerobic SBR supplemented with magnetite under a variety of HRT settings. Steady-state levels were observed in the three SBRs. Under steady-state conditions, above 80% of COD was removed by SBRs with and without magnetite. The microbial Fe (III) reduction resulted in approximately 6% higher COD removal in the SBRs with magnetite. Shorter HRT did not substantially affect the microorganisms' ability to biodegrade COD. Further, experimental results elucidated that stable and enhanced removal of SMX was accomplished in the SBR with 1 g/L magnetite under the HRT of 4, 2, and 1d, with the highest removal efficiency reaching 100%, whereas it was only 60% in the SBR without magnetite. H_2O_2 accumulation in SBRs with magnetite was noticeably higher when HRT exceeded 2 d. H_2O_2 was microbially generated in the sludge flocs and also was released into the bulk phase. Alternating anaerobic/aerobic conditions created continuous oxidation-reduction cycle of magnetite, however, when HRT was lowered to 24 or 12 h, there was a decrease in both the reduced and oxidized Fe in the SBRs containing magnetite. Higher H_2O_2 concentration and larger amount of ferric reduction resulted in greater SMX removal efficiency. These results suggest that the proposed Bio-Fenton SBR with magnetite has long-term operation potential for enhanced treatment of wastewater containing antibiotics. Studies on the removal mechanisms and fundamental reactions occurred during both anaerobic and aerobic periods are needed.

Chapter 4: Removal mechanisms in the Bio-Fenton SBR

4.1 Introduction

Our findings shown in Chapter 3 demonstrated the removal of conventional organic pollutants and SXM was achieved simultaneously in our proposed Bio-Fenton SBR. The mechanisms of the COD removal and the enhanced SMX removal need to be identified. Generally, COD reduction can be primarily attributed to denitrification and methane production under anaerobic conditions, and microbial biodegradation under aerobic conditions. Anaerobic processes are defined as those in the context that take place in the absence of oxygen and nitrate. These anaerobic activities are carried out by a board and diverse group of microorganisms, which often coexist in a mutually beneficial relationship with one another. Despite the fact that the energy circumstances are extremely challenging and, for many bacteria, so unfavorable that it is almost impossible for them to survive, they have in many instances proven to be successful.

Denitrifying bacteria most of which are facultative heterotrophic anaerobes use organic matter in wastewater as the carbon source with 0.47 kg of biomass growth when consuming one kilogram of organic matter (Henze et al., 1995). Methanogens as a group of strict anaerobic microbes, are able to produce methane as a methanolic byproduct under anaerobic circumstances. They prefer using easily biodegradable organic mattes such as acetate, formate, methanol, and methylamines for cell growth and produce methane via methanogenesis. In anaerobic digestion process, about 90% of the energy that is measured as COD present in the substrate can be retrieved in the methane

generated (Henze et al., 1995). The phosphate is utilized by phosphorus accumulation organisms (PAOs) as a source of energy which, under anaerobic circumstances, can be used to take up organic substrate. It was reported that when acetic acid is the substrate, PAOs release 0.2 mol PO_4^{3-} when they take in one mol of acetic acid, and 0.3 kg biomass is produced when one kilogram of acetic acid is consumed (Henze et al., 1995). Therefore, during anaerobic periods, a portion of COD reduction was attributed to P-release. However, since the Bio-Fenton SBR was modified to be exposed under alternative anaerobic and aerobic settings, predominant microbial species probably were selected and changed, resulting in a shift of fundamental reactions that should have happened in the typical anaerobic treatment of wastewater. For instance, IRB utilize the reducing equivalent for ferric reduction under anaerobic conditions and contribute to organic matter decomposition (Lovley & Phillips, 1986). Moreover, many of the bacteria are strictly anaerobic and they are unable to tolerate any amount of oxygen, this is especially true for methane-producing bacteria. Therefore, it is essential to recognize the primal reactions that contributed to COD reduction inside the innovative system.

On the other hand, whether the enhanced removal of SMX observed in the Bio-Fenton SBR was attributed to the biological Fenton reaction initiated by the microbially produced H_2O_2 and magnetite needs to be established. As discussed in Chapter 2, several publications have focused on the Bio-Fenton process and demonstrated its potential to remove toxic organic pollutants that are resistant to other biological processes. However, few of this research on the Bio-Fenton process identified the existence of •OHs formatted via biological Fenton reaction. In this regard, Inagaki et al., 2016 reported the generation of •OHs in aquatic plants in the presence of solid iron compounds. They also observed clear fluorescence emitted from the roots of plants, suggesting the production of •OHs through Bio-Fenton reaction occurred inside the plants.

From these results, it is well reasoned to deduce the occurrence of Bio-Fenton reaction in the SBR supplemented with magnetite.

Because of the short lifespan of •OHs, several different methods have been developed for their identification. The electron-spin-resonance (ESR) method has widespread application in the field of biology for the purpose of directly detecting radicals generated by enzymatic reactions. This technique incorporates the use of a spin trap. Nitrone spin traps generate adducts with lengthy lifetimes and are often employed to detect a wide variety of radicals (Hawkins, 2004). On the other hand, fluorescent probes have also been validated as valuable instruments for detecting •OHs, and they are able to produce temporal (Soh, 2006). Setsukinai et al., 2003 showed the targeted detection of •OHs by employing an aminophenol fluorescein, which could be used to neutrophils that exhibited strong resistance to autoxidation. The application of this method was stated at the cellular level. In this study, it was expected to expand the scope of this application to a macro level in sludge flocs.

4.2 Materials and methods

4.2.1 Chemicals

Fluorescein, 2- [6-(40 -amino)-phenoxy-3H-xanthen-3-on-9-yl]-benzoic acid (APF) with a property of 5 mM N, N-dimethylformamide (DMF) solution was purchased from GORYO Chemical (Sapporo, Japan). The DMF solution was diluted to 10 μM with a 0.1 phosphate buffer of pH 7.4 before use.

4.2.2 Calculations of COD balance

To identify the primal reaction processes during anaerobic and aerobic periods, COD balances were analyzed. The amount of NO_3^--N denitrified, PO_4^{3-}-P released, CH_4 produced, DO consumed and microbial Fe (III) reduced

in anaerobic periods were measured and their corresponding COD removals were calculated according to their stoichiometric relationship to COD reduction (Henze et al., 1995; Lovley & Phillips, 1986), that are, Δ COD/Δ NO_3^--N=5.37 g COD/g NO_3^--N, Δ COD/ΔPO_4^{3-}-P = 4.13 g COD/g PO_4^{3-}-P, Δ COD/Δ CH_4 = 4.00 g COD/g CH_4, Δ COD/Δ DO=1.99 g COD/g DO, and Δ COD/Δ Fe (II) = 0.12 g COD/g Fe (II). The equations used for the calculations are shown in Table 2. NO_3^--N and PO_4^{3-}-P concentrations in the supernatant were measured using an ion chromatography system (Dionex ICS-2100 Thermo Scientific). The produced gas was collected using gas collection bag with the volume of 1 L and sampled by a syringe, and CH_4 content was analyzed using a gas chromatograph (GC-2010 Shimadzu).

Table 2. Reaction equations and references for calculating the stoichiometric relationship between different processes and COD reduction.

$$0.61C_{18}H_{19}O_9N + 4.54NO_3^- + 0.39NH_4^+ + 4.15H^+ \rightarrow C_5H_7NO_2 + 2.27N_2 + 5.98CO_2 + 5.15H_2O$$	Δ COD/Δ NO_3^--N=5.37 g COD/g NO_3^--N
$$C_{18}H_{19}O_9N + 17.5O_2 + H^+ \rightarrow 18CO_2 + 8H_2O + NH_4^+$$	
$$2C_2H_4O_2 + (HPO_3) + H_2O \rightarrow (C_2H_4O_2) + PO_4^{3-} + 3H^+$$	Δ COD/ΔPO_4^{3-}-P = 4.13 g COD/g PO_4^{3-}-P
$$C_2H_4O_2 + 2O_2 \rightarrow 2CO_2 + 2H_2O$$	
$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$	
Lovley and Phillips, 1986 investigated the quantities of CH_4 and Fe (II) produced in sediments with and without additional Fe (III) to explore the conversion of electron flow from CH_4 production to Fe (III) reduction. The findings indicated that for every 9.28 units of Fe (II) production, there was a 0.98 unit decrease in CH_4 production.	Δ COD/Δ CH_4 = 4.00 g COD/g CH_4 Δ COD/Δ Fe (III) = 0.12 g COD/g Fe (III)
Where, $C_{18}H_{19}O_9N$ indicates the chemical composition of organic matter in wastewater, $C_5H_7NO_2$ indicates the chemical composition of biomass in activated sludge.	(Henze et al., 1995)

4.2.3 Detection of hydroxyl radicals

In order to identify the formation of hydroxyl radicals, a novel fluorescence reagent, APF was used, and fluorescence was recorded using a confocal laser scanning microscopy (FV-1000D, Olympus). APF can specifically identify particular types of highly reactive oxidative species (hROS) by an increase of fluorescence and exhibit perfect resistance to autoxidation in vitro and in vivo. It was reported that almost non-fluorescent APF would undergo *O*-dearylation upon reaction with rROS such as ·OHs to yield fluorescent fluorescein with a strong emission spectrum (Setsukinai et al., 2003). The reaction scheme is shown in Figure 15. In experiments, concentrated APF solution containing 5 mM DMF was diluted before being used to incubate the samples. 50 μL original APF solution was added into 20 mL aerated buffer solution (formula is shown in Table 3). One milliliter of a mixture of sludge and magnetite was withdrawn from each reactor during the aerobic periods and subsequently incubated in 10 mL diluted APF solution (containing 10 μM DMF) in 10 mL beakers. Small magnetic stirrers were used to ensure sufficient contact between sludge flocs and APF. The incubation period was two days under aerobic conditions. The incubated mixture was stimulated for 10 min at a wavelength of 490 nm and viewed through a 515 nm emission filter. To trace the fluorescence of ·OHs, an Olympus BX60 equipped with a digital microscope camera (MoticamS1, Motic, Amoy, China) was employed. Image processing was performed using Motic Images Plus 2.4S (Inagaki et al., 2016).

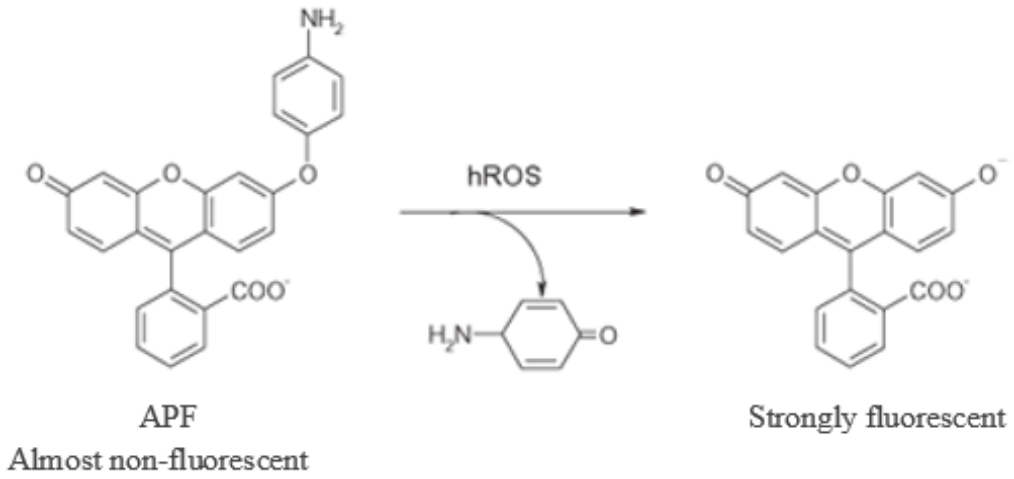

Figure 15. Scheme of O-dearylation reaction of APF with hROS (Setsukinai et al., 2003).

Table 3. Compositions of the buffer solution used to dilute APF solution.

Chemical	KH_2PO_4	KCl	Na_2HPO_4	NaCl
Concentration (mg/L)	200	200	1150	8000

4.3 Results

4.3.1 Anaerobic COD balances

Primary reactions in both aerobic and anaerobic environments were examined to better understand the mechanisms of COD removal. From Figure 16(a), COD was removed during both anaerobic and aerobic periods. To determine which activities principally contribute to the anaerobic COD removal, processes including denitrification, phosphate release, CH_4 production, DO consumption, and dissimilatory Fe (III) reduction were assessed. Because the influent was not a completely anaerobic environment, an oxygen consumption process would occur in the beginning of an anaerobic period. After exchanging the synthetic wastewater, SBRs presented a temporary anoxic environment, the dissolved oxygen was later consumed by microorganisms to create a strictly anaerobic environment. Under the steady-state conditions in Phases II, III, IV, and V, the amount of NO_3^--N, PO_4^{3-}-P, CH_4 production, and DO, were monitored at the beginning and end of four continuous anaerobic period. Their average observed removals are shown in Table S1 in Appendix.

Figure 16(b) and (c) illustrate the time course variations of COD, NO_3^--N and PO_4^{3-}-P of three SBRs during anaerobic periods. In Phase II three SBRs had remarkable similar NO_3^--N removals with values of around 11 mg/L; however, when HRT was lowered, the quantity of nitrate denitrified became smaller with values of 3, 7, and 2 mg/L in SBR0, SBR1, and SBR2, respectively [Figure 16(b)]. The major explanation for this was that during the aerobic period, the amount of nitrate that was created via nitrification was much lower with shorter HRT, which resulted in their low concentrations at the beginning of the anaerobic period. The greater NO_3^--N concentration and almost no detection of NO_3^--N in SBR1 after aerobic and anaerobic periods, respectively, implies that the addition of magnetite at a concentration of 1 g/L not only did not inhibit

nitrification and denitrification processes, but actually accelerated the activity of the essential bacteria involved in these two processes. From Figure 16(c), PO_4^{3-}-P in three SBRs increased and decreased under anaerobic and aerobic conditions, respectively. The unusual phenomenon in Phase II was due to the fact that phosphate nutrient was not added into the influent. After adding 20 mg/L of PO_4^{3-}-P to the synthetic wastewater, reliable phosphate release and uptake process was observed during anaerobic and aerobic period, respectively. The amount of phosphorus up taken aerobically was greater than that released anaerobically, suggesting the ability of the Bio-Fenton SBR to remove nutrients such as phosphorus was unaffected while effectively removing antibiotics.

During Phase II, the measured amounts of methane produced in SBR0, SBR1, and SBR2 were about 0.47, 0.48, and 0.47 mg/L, respectively (Table S1 in Appendix). Lovley and Phillips, 1986 observed that ferric reduction process inhibited methane generation by 50-90% in anaerobic sediments environment because IRB competed with methanogens for electron donors and shift electron flow away from methanogens. However, methanogens are primarily anaerobic microorganisms, they would not function and survive under aerobic periods. *Candidatus Mehanothrix Paradoxum*, a species of methanogens that has been discovered as able to tolerate oxygen stress for a prolong time may be responsible for the extremely small quantity of methane detected in Phase II (Peters and Conrad, 1995). However, there was no evidence of any methane production when the HRT was less than 4 days.

In accordance with the stoichiometric relationships discussed in Section 4.2.2, the corresponding COD removals caused by denitrification, phosphate release, CH_4 production, DO consumption, and dissimilatory Fe (III) reduction have been calculated and are presented in Table S1 of the Appendix. Figure 17 provides a concise summary of the proportion of COD removal contributed by these reactions to the overall anaerobic COD removal. In these three SBRs,

under the HRT of 4 and 2 days (Phase II and III), denitrification consistently supplied the largest percentage toward anaerobic COD removal. Phosphate release came in second place, with the exception of Phase II. The absence of a phosphate supply in the influent in Phase II was the root of this issue. While in the SBRs with magnetite, the COD removal contributed by microbial ferric reduction ranked third. In SBR0 and SBR2, when the HRT was reduced from 4 days to 12 hours, the proportion of anaerobic COD removal that was provided by denitrification dropped from 79 to 15% and from 61 to 14%, respectively. When the HRT was 12 days, microbial Fe (III) reduction was the second major contributor to anaerobic COD removal, accounting for 17% and 19% in SBR1 and SBR2, respectively. Along with the drop in HRT, there was also a decrease in the contribution made by microbial Fe (III) reduction in SBR1 and SBR2. However, when HRT was lowered to 24 and 12 hours (Phase IV and V), the proportion of anaerobic COD removal that was provided by the unknown mechanism rose up to even more than half, especially in SBR0. This was due to, on one hand, the decreased amount of denitrified nitrate and reduced ferric in these two phases. On the other hand, in regard to the unidentified processes, from the fast-growing MLVSS values displayed in Figure 8, it was speculated that microorganisms in the sludge consumed organic matter to synthesize biopolymers, which are crucial for the storage of energy and the production of biofilm. As discussed in Section 3.3.2.1, in the latter three phases, SBR0 showed higher MLSS and MLVSS compared to SBR1 and SBR2, leading to the inference that the added magnetite affected the ability of microorganisms to take up organic matter for the synthesis of biopolymers. This may also help to explain why an unidentified mechanism in SBR0 supplied a greater proportion of the anaerobic COD removal. Li et al., 2021 recovered extracellular biopolymers from conventional activated sludge in the range of 90 to 190 mg/g VSS, and it was shown that an increase in the number of soluble organics present in the influent might promote the formation of biopolymers. In the

synthetic wastewater supplied to the SBRs in this study, glucose, sodium acetate, urea, and peptone were the main organic nutrients, which were readily utilized by bacteria to synthesize biopolymers. Therefore, it was assumed that bacteria consuming organic substrates to synthesize polymers were responsible for the portion of the anaerobic COD removal that was not provided by the mechanisms that had been recognized. Nevertheless, it is strongly suggested that future investigations for detecting the synthesis of biopolymers.

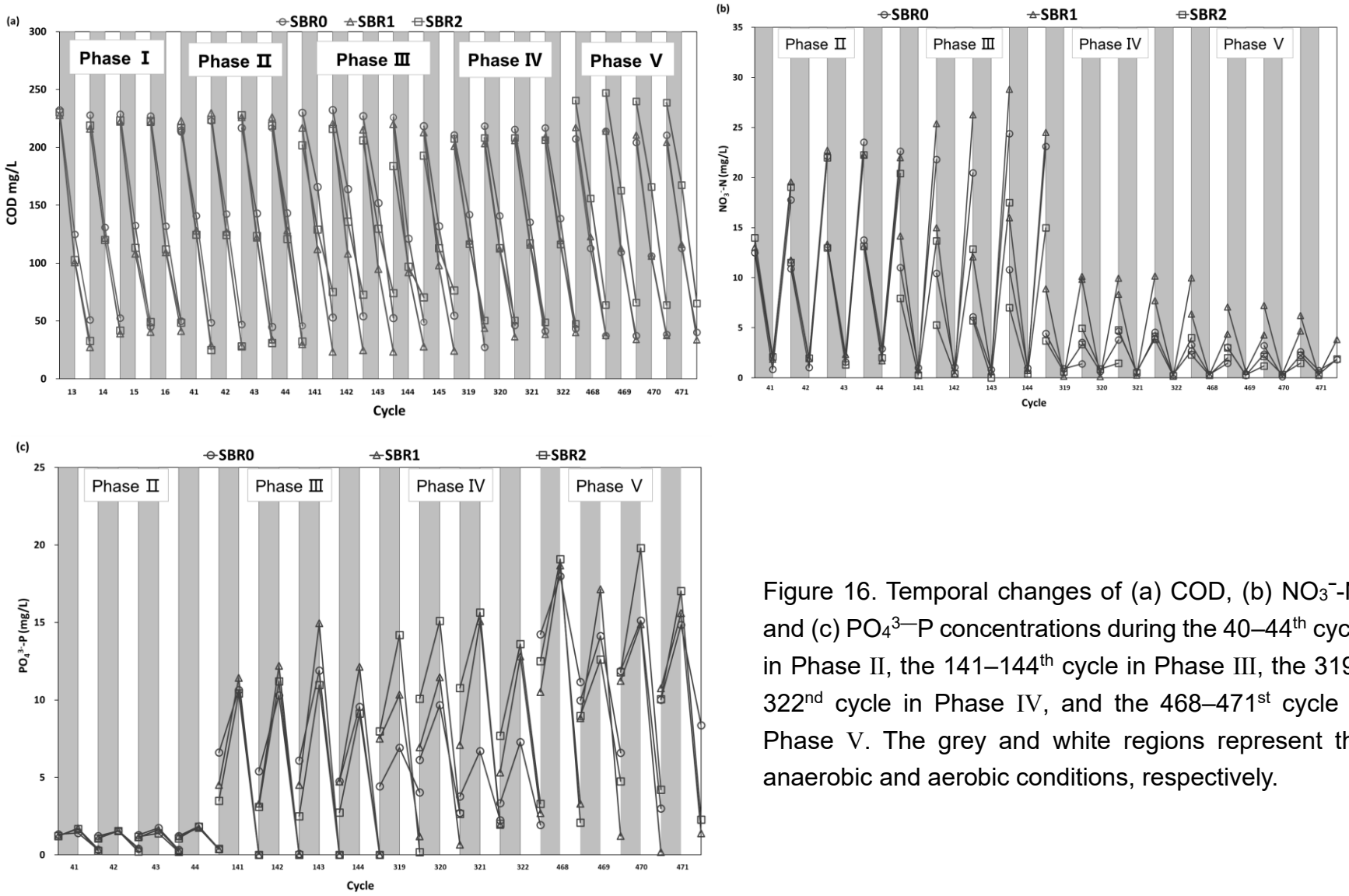

Figure 16. Temporal changes of (a) COD, (b) NO_3^--N, and (c) PO_4^{3-}P concentrations during the 40–44th cycle in Phase II, the 141–144th cycle in Phase III, the 319–322nd cycle in Phase IV, and the 468–471st cycle in Phase V. The grey and white regions represent the anaerobic and aerobic conditions, respectively.

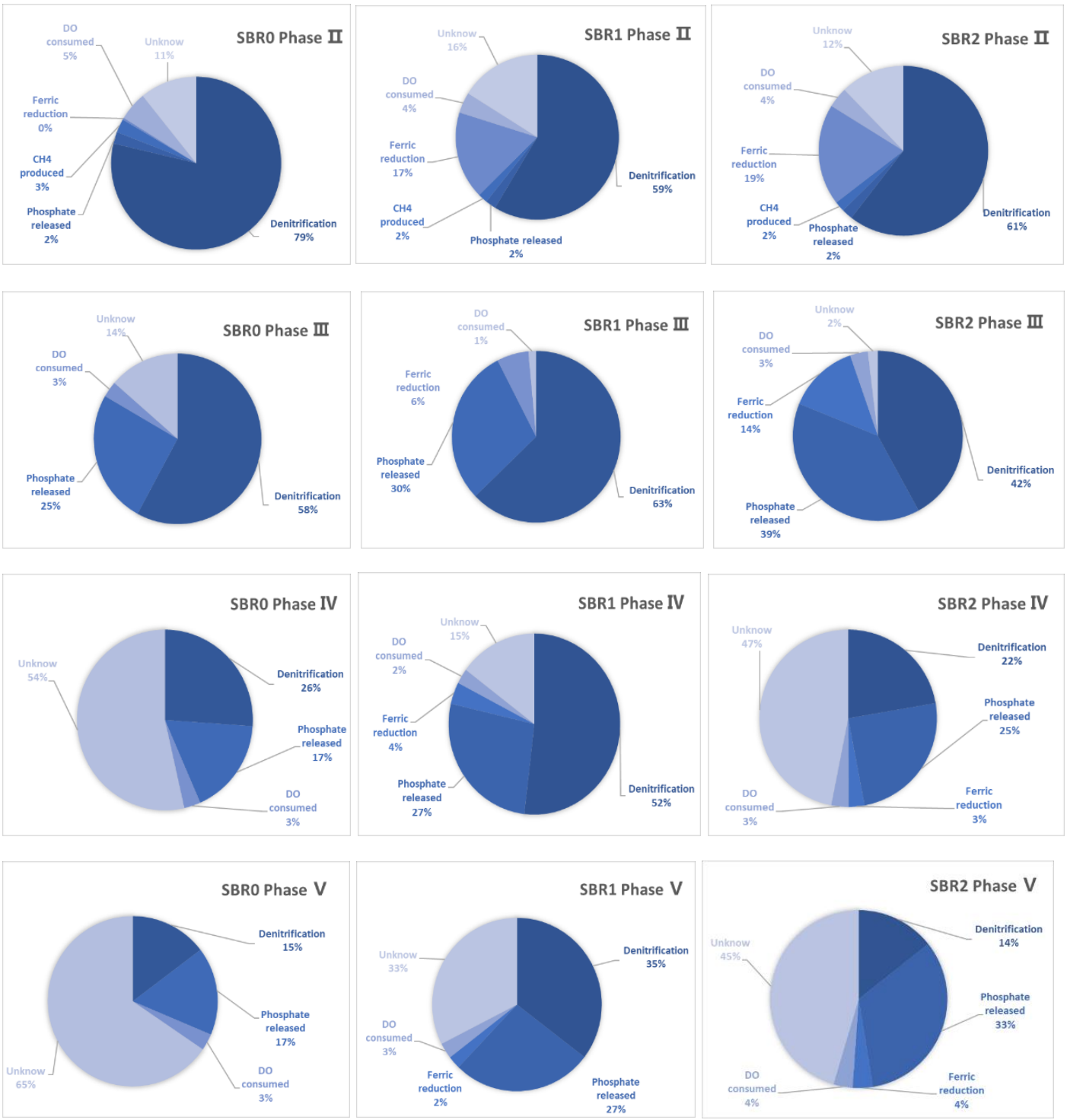

Figure 17. The proportion that different processes contributed to the overall anaerobic COD.

4.3.2 Biological Fenton process

To identify the enhanced SMX removal in the SBRs supplemented with magnetite was attributed to the biological Fenton reaction, the formation of ·OHs was detected. Fluorescence probes are effective for detecting ·OHs and providing temporal and spatial images. APF, as a selectable fluorescein, can be used by neutrophils with high resistance to autoxidation. Sludge flocs were taken from each SBR during aerobic period, after the incubation of the samples, their images were captured under visible-light irradiation and fluorescent light, before being compared in Figure 18. As shown in the images of SBR1 and SBR2 in Phases II and III, strong fluorescent emissions were found in the matrix of magnetite-sludge aggregates, implying the creation of ·OHs and the occurrence of biological Fenton reactions. However, under fluorescent irradiation, extremely weak fluorescence was observed in the sample from SBR0 in Phase II. This may be due to the slight presence of iron compounds in the seed sludge, which reacted with H_2O_2 (Figure 11), and produced a limited amount of ·OHs. No fluorescence could be seen in the samples from both SBR1 and SBR2 in Phase V, because of the very low H_2O_2 concentration (Figure 11) and the inadequate redox of magnetite [Figure 13(b) and (c)], leading to a reduced amount of SMX removal (Figure 10).

The same observations were made for the activated sludge under anaerobic conditions, whereas no clear fluorescent emissions were observed (data not shown). This also explained that SMX was mainly removed during aerobic periods. In SBR1 and SBR2, magnetite catalyzed the oxidation of the microbially produced H_2O_2 and generated multiple ·OHs with strong potential to decompose SMX during aerobic periods. After the systems reached a steady state, the continuous production of H_2O_2 during aerobic periods, and the reduction of magnetite during anaerobic periods contributed to the continued

occurrence of the biological Fenton reaction. This resulted in stable and enhanced SMX removal in the SBRs containing magnetite in certain phases. In Phases II, the productions of ·OHs in SBR1 and SBR2 were supposed to be comparable, since the fluorescence intensities observed in their matrixes of magnetite-sludge aggregates were similar and almost identical SMX removals were observed in these two SBRs (Figure 10). On the other hand, in Phase III, it was noticed that SBR2 had a weaker fluorescence compared to SBR1, which may be explained by the fact that SBR2 had a lower H_2O_2 concentration (Figure 11).

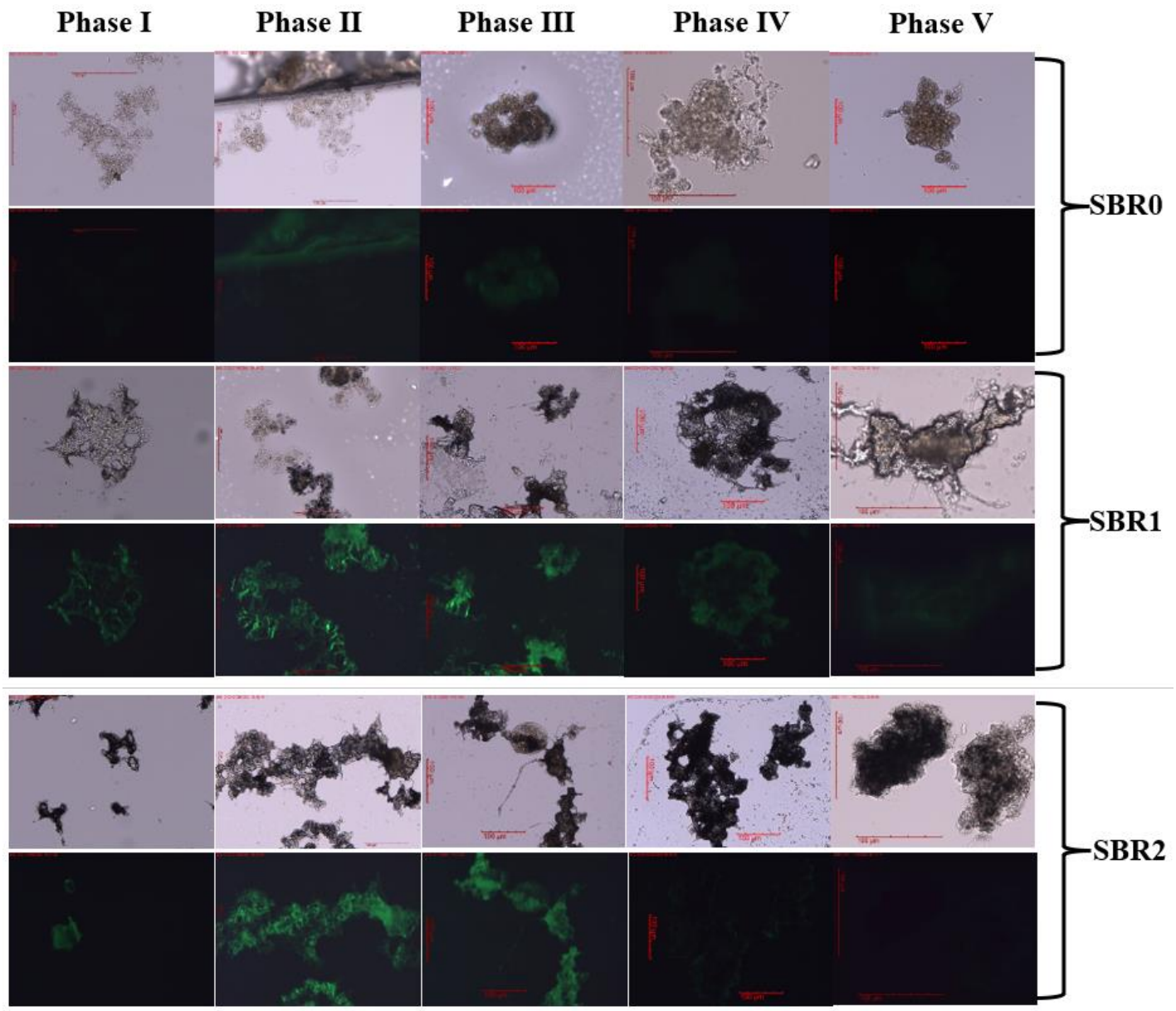

Figure 18. Identification of ·OHs by fluorescence microscopy. The upper images for each SBR were taken with visible-light; the lower images for each SBR were taken with fluorescent-light. The bars in the images indicate a scale of 100 μm.

4.4 Conclusions

In this chapter, the mechanisms of both COD and SMX removal were explored, especially in terms of anaerobic COD removal and the enhanced SMX removal in the Bio-Fenton SBR. COD balances were analyzed by identifying the stoichiometric relationships between several common anaerobic reactions and their corresponding COD reductions. In the Bio-Fenton SBR supplemented with 1 g/L of magnetite, denitrification always supplied the highest proportion toward anaerobic COD removal. Phosphate release came in second place. Compared to the control SBR and Bio-Fenton SBR with 3 g/L of magnetite, the presence of magnetite at a concentration of 1 g/L not only did not inhibit nitrification and denitrification processes, but actually improved the activity of the key bacteria engaged in these two processes. Along with the decrease in HRT, there was also a decrease in the contribution to COD reduction caused by microbial Fe (III) reduction in SBR1 and SBR2. The consumption of organic matter by microorganisms, followed by the synthesis of biopolymers, was thought to be the unidentified mechanism responsible for the anaerobic COD removal. The enhanced SMX removal in the Bio-Fenton SBRs during particular phases was revealed to be due to the formation of ·OHs through the biological Fenton reaction. These discoveries were made using a fluorescence-microscopy observation. Fluorescence mainly was observed in the sludge flocs, indicating the Bio-Fenton reaction took place in the matrix of magnetite-sludge aggregates. The intensity of the fluorescence observed in the Bio-Fenton SBRs showed a positive association with H_2O_2 concentration as well as the extent of redox of magnetite.

Chapter 5: Conclusions and future studies

The treatment of emerging organic contaminants (EOCs) has attracted great attention recently because of the harm they have already caused to the aquatic environment and human health. Antibiotics as one of the most often used classes of EOCs, have caused severe issues with antimicrobial resistance (AR). Biological process-based conventional wastewater treatment plants (WWTPs) have limited capacity to treat such refractory trace contaminants to an acceptable level, thus more efficient techniques are urgently required. Advanced oxidation processes (AOPs) are considered a very promising approach for resolving this issue because the generated hydroxyl radicals (·OHs) can react non-selectively with a wide range of organic contaminants. Among them, Fenton-based process is the most intensively studied and employed. Due to a number of shortcomings of the classic Fenton reaction, a series of modified Fenton processes (e.g., heterogeneous Fenton reaction) and combined biological treatment and AOPs have been studied by an increasing number of researchers. However, limitations such as the constant requirement for Fenton reagents (e.g., H_2O_2) still have not been overcome. Regarding a much more effective and sustainable technique, some published research on the topic of producing H_2O_2 in-situ were examined, such as by making use of bacteria. Some scholars have investigated the biological synthesis of H_2O_2 and utilized it in the Fenton process; this process is referred to as Bio-Fenton reaction. Based on this knowledge, this study was undertaken to construct a Bio-Fenton sequencing batch reactor (SBR) that would allow the mixed microbial cultures existing in the activated sludge to produce H_2O_2. The added magnetite had the potential to catalyze the oxidation of H_2O_2 and initiated a biological Fenton reaction. The investigation into the simultaneous removal of COD and the model antibiotic sulfamethoxazole (SMX) was carried out through

the long-term operation of the Bio-Fenton SBRs. Furthermore, the fundamental removal mechanisms in the Bio-Fenton SBR have been uncovered. Based on the experimental results, the main conclusions can be drawn as follows,

1) Stable and enhanced SMX removal was achieved in the Bio-Fenton SBR with 1 g/L of magnetite under the hydraulic retention time (HRT) of 4, 2, and 1 day, with the highest removal efficiency reaching 100%, which was 40% higher than that in the SBR without magnetite. The removal of COD and nutrients (including nitrogen and phosphorus) was unaffected by the inclusion of magnetite, and the SBRs with and without magnetite were all able to attain above 80% of COD removal.

2) H_2O_2 accumulation in the Bio-Fenton SBR with 1 g/L of magnetite was distinctively higher. In addition to being produced by microbes in the sludge flocs, hydrogen peroxide was also released into the bulk phase. Alternating anaerobic/aerobic conditions resulted in the continuous redox cycle of magnetite, allowing the biological Fenton reaction to occur continuously.

3) Denitrification, phosphorus release, and microbial ferric reduction were the primary mechanisms of anaerobic COD removal in the SBR supplemented with 1 g/L of magnetite. The enhanced SMX removal in the Bio-Fenton SBRs during particular phases was revealed to be due to the formation of ·OHs through biological Fenton reaction, which took place in the matrix of magnetite-sludge aggregates.

Overall, this study strengthens the idea that stable and enhanced removal of antibiotics by integrating activated sludge process with AOPs in an SBR supplemented with magnetite can be achieved. The insights gained from this research may be of assistance to develop a notably more effective, economical, and sustainable method for treating wastewater containing organic contaminants of emerging concern. Despite the fact that this study only examined the removal of one type of antibiotic, the detection of hydroxyl radicals proved that this method will also be effective for removing other types

of antibiotics. Another limitation of this study is that more ranges of different magnetite dosages, HRTs, or organic loadings need to be investigated before the optimal operational condition can be determined. Since there have been no studies concentrating on the development of a model that is suitable for the Bio-Fenton process, where microbial production of H_2O_2 and the redox cycle of magnetite are included, therefore, for future research on the kinetic study, the Bio-Fenton process needs to be modeled appropriately to optimize the operating conditions. Furthermore, considerably more work will need to be done to quantify the abundance of bacteria and archaea in the Bio-Fenton SBRs to provide much more precise mechanisms as the addition of magnetite and the alternative anaerobic/aerobic conditions would cause a shift in the predominant bacterial species. Although enhanced SMX removal was observed in this research, the intermediates and byproducts that were formed during the biodegradation of SMX need to be recognized.

Appendix

The decrease of SMX during anaerobic periods

During Phase I and II, after anaerobic periods, SMX concentrations were examined, and the decreases in SMX concentrations in anaerobic periods were shown in Figure S1. It can be noted that less 0.13 mg/L of SMX was removed during anaerobic periods, which contributed relatively little to the overall removals (Figure 10).

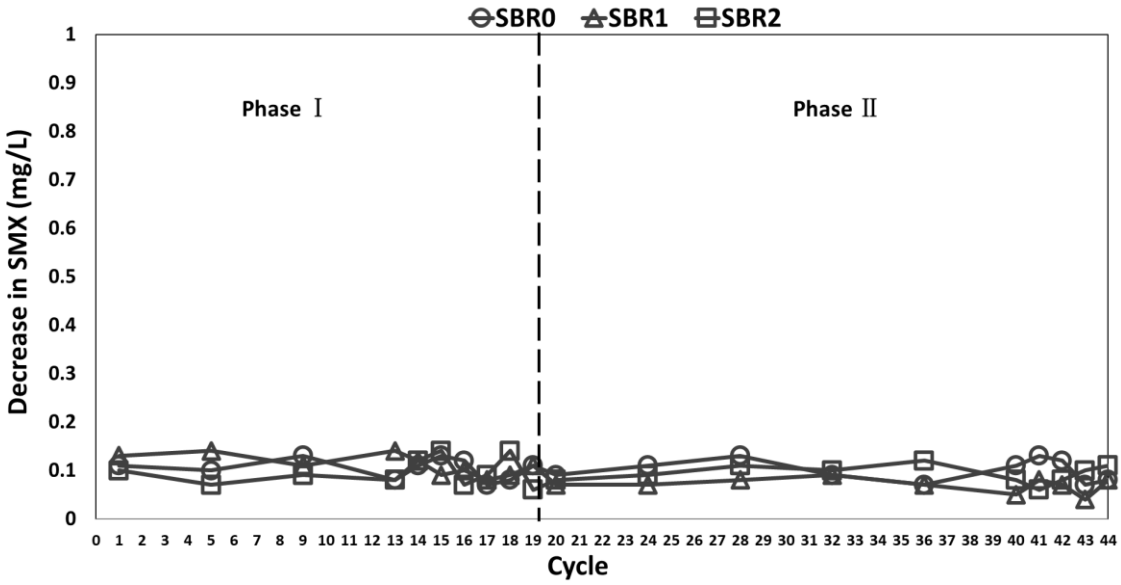

Figure S1. The decrease in SMX of SBR0, SBR1, and SBR2 during anaerobic period of the cycle.

SMX adsorption experiment

Preliminary batch experiment was conducted to investigate the adsorption of SMX on magnetite. The composition of the synthetic wastewater was the same as that used for the SBR experiment. SMX and magnetite concentration was set as 1 mg/L and 3 g/L, respectively. The experiment was conducted for 6 days including a 3-day anaerobic period and a 3-day aerobic period. The effective volume was 100 mL. Samples were withdrawn at predetermined time intervals. Figure S2 shows the change in adsorbed SMX on magnetite. It was found that about 8% of SMX was removed by adsorption, which was much smaller than removal efficiencies in the SBR experiments (Figure 10).

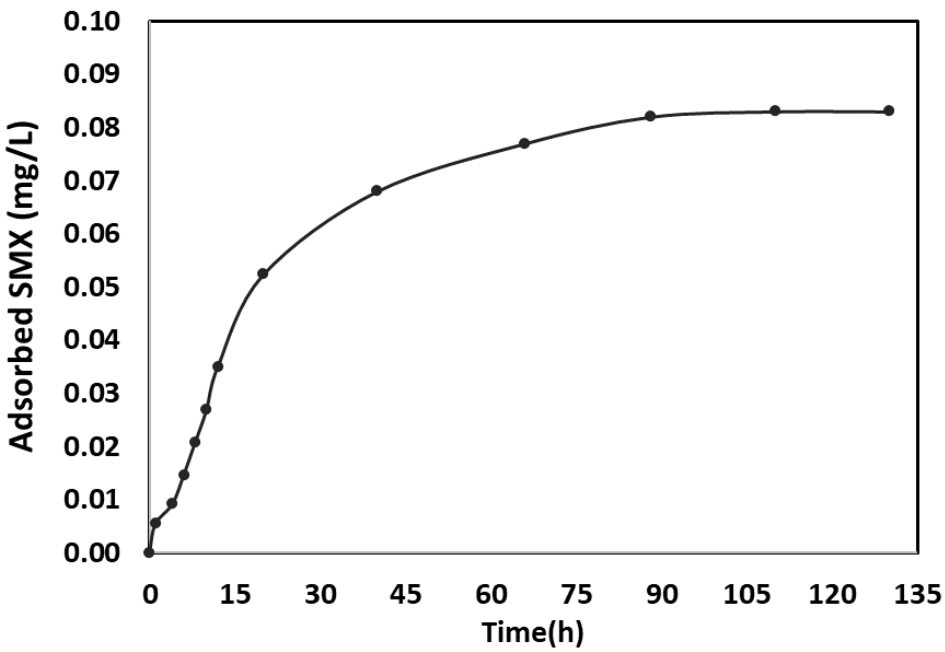

Figure S2. The profile of the concentration of SMX absorbed on magnetite.

Table S1. The summarization of the COD balances during certain anaerobic periods in Phase II, Phase III, Phase IV, and Phase V.

Phase II	Parameter	Amount (mg/L) [a]	Corresponding COD removal (mg/L) [a]	Observed anaerobic COD removal (mg/L) [a b]	Anaerobic COD balances
SBR0	NO_3^--N denitrified	11±0.7	59.1±3.8	75.1±3.1	78.7%
	PO_4^{3-}-P released	0.4±0.2	1.7±0.8		2.2%
	CH_4 produced	0.5±0.02	1.9±0.1		2.6%
	Fe (III) reduced	2.3±0.6	0.3±0.1		0.4%
	DO consumed	2.0±0.2	4.1±0.3		5.4%
	Unknow	-	-		10.8%
SBR1	NO_3^--N denitrified	10.9±0.6	58.5±3.2	99.8±3.6	58.7%
	PO_4^{3-}-P released	0.5±0.0	2.1±0.0		2.1%
	CH_4 produced	0.5±0.02	1.9±0.1		1.9%
	Fe (III) reduced	143.3±25.3	17.2±4.2		17.2%
	DO consumed	2.0±0.07	4.1±0.1		4.1%
	Unknow	-	-		16.1%
SBR2	NO_3^--N denitrified	11.1±0.9	59.6±4.8	99.5±4.4	60.5%
	PO_4^{3-}-P released	0.5±0.2	2.1±0.8		2.1%
	CH_4 produced	0.5±0.01	1.9±0.04		1.9%
	Fe (III) reduced	158.3±55.9	19.0±6.7		19.3%
	DO consumed	1.9±0.4	3.8±0.9		3.9%
	Unknow	-	-		12.3%
Phase III	**Parameter**	**Amount (mg/L) [a]**	**Corresponding COD removal (mg/L) [a]**	**Observed anaerobic COD removal (mg/L) [a b]**	**Anaerobic COD balances**
SBR0	NO_3^--N denitrified	8.6±1.9	46.2±10.2	79.8±14.7	57.9%
	PO_4^{3-}-P released	4.9±0.6	20.2±2.5		25.3%
	Fe (III) reduced	0.6±2.5	0.1±0.3		0.1%
	DO consumed	1.2±0.2	2.4±0.4		3.0%
	Unknow	-	-		13.7%

SBR1	NO_3^--N denitrified	13.7±1.5	73.6±8.1	116.2±7.8	63.3%
	PO_4^{3-}-P released	8.4±1.4	34.7±5.8		29.9%
	Fe (III) reduced	59.3±20.1	7.1±2.4		6.1%
	DO consumed	0.9±0.2	1.8±0.4		1.7%
	Unknow	-	-		-0.1%
SBR2	NO_3^--N denitrified	6.2±1.0	59.6±5.4	79.2±4.7	42.0%
	PO_4^{3-}-P released	7.5±0.8	31.0±3.3		39.1%
	Fe (III) reduced	89.5±39.3	19.0±4.7		13.6%
	DO consumed	1.4±0.5	3.8±1.0		3.4%
	Unknow	-	-		1.9%
Phase IV	**Parameter**	**Amount (mg/L) [a]**	**Corresponding COD removal (mg/L) [a]**	**Observed anaerobic COD removal (mg/L) [a b]**	**Anaerobic COD balances**
SBR0	NO_3^--N denitrified	3.7±0.5	19.9±2.7	76.2±4.5	26.1%
	PO_4^{3-}-P released	3.2±0.6	13.2±2.5		17.3%
	Fe III) reduced	0.0±0.0	0.0±0.0		0.0%
	DO consumed	1.2±0.1	2.3±0.2		3.0%
	Unknow	-	-		53.6%
SBR1	NO_3^--N denitrified	8.5±0.8	45.6±4.3	88.0±3.6	51.9%
	PO_4^{3-}-P released	5.7±2.1	23.5±8.7		26.8%
	Fe (III) reduced	28.7±6.7	3.4±0.8		3.9%
	DO consumed	1.2±0.4	2.4±0.8		2.7%
	Unknow	-	-		14.8%
SBR2	NO_3^--N denitrified	3.8±0.5	20.4±2.7	91.7±2.0	22.3%
	PO_4^{3-}-P released	5.5±0.6	22.7±2.5		24.8%
	Fe (III) reduced	22.3±5.2	2.7±0.6		2.9%
	DO consumed	1.9±0.4	3.8±0.9		3.9%
	Unknow	-	-		46.9%
Phase V	**Parameter**	**Amount (mg/L) [a]**	**Corresponding COD removal (mg/L) [a]**	**Observed anaerobic COD removal (mg/L) [a b]**	**Anaerobic COD balances**

SBR0	NO_3^--N denitrified	2.7±0.5	14.5±2.7	98.8±3.6	14.7%
	PO_4^{3-}-P released	4.0±0.6	16.5±2.5		16.7%
	Fe (III) reduced	0.0±0.0	0.0±0.0		0.0%
	DO consumed	1.6±0.03	3.1±0.1		3.2%
	Unknow	-	-		65.4%
SBR1	NO_3^--N denitrified	6.4±0.4	34.4±2.1	97.2±6.4	35.4%
	PO_4^{3-}-P released	6.3±2.0	26.0±8.3		26.8%
	Fe (III) reduced	19.7±2.2	2.4±0.3		2.4%
	DO consumed	1.4±0.1	2.9±0.2		2.9%
	Unknow	-	-		32.4%
	NO_3^--N denitrified	2.1±0.3	11.3±1.6	78.6±6.1	14.3%
	PO_4^{3-}-P released	6.3±1.6	26.0±6.6		33.1%
SBR2	Fe (III) reduced	24.0±6.2	2.9±0.7		3.7%
	DO consumed	1.4±0.04	2.7±0.1		3.5%
	Unknow	-	-		45.4%

[a] mean ± standard deviation (n=4)

[b] the difference between initial and final concentration of anaerobic period in each cycle

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List of publications

1. Enhanced sulfamethoxazole removal using an anaerobic and aerobic sequencing batch reactor with magnetite, *Engineering for Rural Development*, 2021, doi: 10.22616/ERDev.2022.21.TF026. **T. Shen**, Y. Inagaki, H. Koike, R. V. Pariyarath, M. Komori, and Y. Sakakibara

2. Enhanced removal of sulfamethoxazole by an anaerobic/aerobic SBR with an oxidation-reduction cycle of magnetite, *Journal of Water Process Engineering*, vol. 48, 2022, doi: 10.1016/j.jwpe.2022.102817. **T. Shen**, Y. Inagaki, M. Komori, and Y. Sakakibara

Conference papers and presentations

1. Enhanced sulfamethoxazole removal using an anaerobic and aerobic sequencing batch reactor with magnetite, The 4th International Symposium on Water Resource and Environmental Management (WREM 2021), Sanya, China, 2021, **T. Shen**, Y. Inagaki, H. Koike, R. V. Pariyarath, M. Komori, and Y. Sakakibara

2. Enhancement of sulfamethoxazole removal in an anaerobic/aerobic sequencing batch reactor with magnetite, 56th Annual Conference of JSWE, Toyama, Japan, 2022, **T. Shen**, Y. Inagaki, M. Komori, and Y. Sakakibara

3. Sulfamethoxazole and COD removal by a novel anaerobic/aerobic SBR supplemented with magnetite, Poster, IWA World Water Congress & Exhibition, Copenhagen, Denmark, 2022, **T. Shen**, Y. Inagaki, M. Komori, and Y. Sakakibara

4. Effect of HRT on the Removal of Sulfamethoxazole in a Bio-Fenton SBR, 57th Annual Conference of JSWE, Matsuyama, Japan, 2023, **T. Shen**, M. Komori, and Y. Sakakibara